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VOL. 30 JULY, 1945 No. 7

The Journal of Laboratory and Clinical Medicine

Clinical and Experimental

Immunologic Studies in Patients With Subacute Bacterial Endocarditis Treated by Combined Penicillin-Heparin Method. I. Sensitivity to Penicillin. Max Grolnick, M.D., and Leo Loewe, M.D., Brooklyn, N. Y. ---- 559

The Effect of Artificially Induced Fever on Humoral Antibodies and on Histamine Intoxication in the Guinea Pig. R. Y. Gottschall, Ph.D., D. Laurent, B.S., and P. deKruif, Ph.D., Lansing, Mich. In Collaboration With W. M. Simpson, M.D., H. W. Kendell, M.D., and D. L. Rose, M.D., Dayton, Ohio 563

Studies on Rickettsial Agglutination in Typhus. Florence K. Fitzpatrick, M.A., Glenolden, Pa. ---- 577

The Behavior of Blood Cholesterol in Thyrotoxic Patients Under Treatment With Thiouracil. T. H. McGavack, B.A., M.D., F.A.C.P., and I. J. Drekter, B.S., New York, N. Y. ---- 586

A Note on the In Vitro Respiration of Muscle in Myasthenia Gravis. F. J. Stare, M.D., Boston, Mass., and H. T. Ricketts, M.D., Chicago, Ill. ---- 589

The Effect of Insulin on the In Vitro Respiration of Human Skeletal Muscle. Henry T. Ricketts, M.D., Chicago, Ill., and F. J. Stare, M.D., Boston, Mass. 594

The Effect of Ultraviolet Irradiation on the Blood Hemoglobin. Adelaide P. Barer, Ph.D., and Willis M. Fowler, M.D., Iowa City, Iowa ---- 600

The Effect of Digitalis, Epinephrine, and Surgery on the Response to Heparin. Campbell Moses, M.D., Pittsburgh, Pa. ---- 603

Laboratory Methods

A Demand Apparatus for Automatic Delivery of Aerosols During Inspiration. Morris Eckman, B.S., Charles C. Rumsey, Jr., Beatrice Barach, and Alvan L. Barach, M.D., New York, N. Y. ---- 608

(Contents continued on inside cover)

PUBLISHED BY THE C. V. MOSBY COMPANY, 3207 WASHINGTON BLVD., ST. LOUIS 3

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CONTENTS—(*Cont'd*)

Determination of Hemoglobin. John T. Van Bruggen, Ph.D., St. Louis, Mo. ---- 611

The Determination of the Free and Total Cholesterol of Plasma With the Photoelectric Colorimeter. Delphine H. Clarke, M.D., and Amy F. Marney, B.S., New York, N. Y. ---- 615

Simplification of U. S. P. XII Microbiological Vitamin Assays. William F. Elias, Ph.D., Joseph Merrion, M.S., Anne Nagler, B.S., and Marion Broome, Philadelphia, Pa. ---- 622

An Optical Fluorescence Comparator. Kurt Lange, M.D., New York, N. Y., Commander Milton J. Matzner, Medical Corps, United States Naval Reserve, and Semyon E. Krewer, New York, N. Y. ---- 627

A Method for Determining the Coagulation Time Using Capillary Blood. T. K. Kruse, Ph.D., and Campbell Moses, M.D., Pittsburgh, Pa. ---- 631

A Simple Procedure for Determining the Approximate Concentration of Pepsin in Gastric Contents. Israel S. Kleiner, Ph.D., New York, N. Y. ---- 634

Book Reviews

Book Reviews ---- 636

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Vol. 30, No. 7, July, 1945. The Journal of Laboratory and Clinical Medicine is published monthly by The C. V. Mosby Company, 3207 Washington Blvd., St. Louis (3), Mo. Subscription Price: United States, its Possessions, Pan-American Countries, $8.50 per year; Canada, $9.50; Foreign, $9.50. Entered as Second-Class Matter at Post Office, at St. Louis, Mo., under Act of March 3, 1879. Printed in the U. S. A.

IMMUNOLOGIC STUDIES IN PATIENTS WITH SUBACUTE BACTERIAL ENDOCARDITIS TREATED BY COMBINED PENICILLIN-HEPARIN METHOD

I. Sensitivity to Penicillin

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THIS series of studies was undertaken to investigate factors causing untoward reactions in patients who were undergoing therapy with a combination of penicillin and heparin.[1-3] The purpose of the present investigation was to determine whether the patients receiving intensive and prolonged treatment with penicillin developed a sensitivity to the drug, and whether they eventually acquired a sensitivity to the mold, *Penicillium notatum,* the source of penicillin.

The procedure of combined penicillin-heparin treatment of patients with subacute bacterial endocarditis consists of the continuous intravenous administration of penicillin in saline solution for a sufficient period of time to render the blood sterile. In addition, heparin, in a retarding menstruum,[4] is administered subcutaneously at intervals of several days, or the aqueous solution is given with the infusion of penicillin. Generally a single course of treatment is adequate, although some patients require repeated courses.

In a rapidly expanding literature on the results of penicillin therapy, there is little information pertaining to allergic phenomena due to the drug or to immunologic studies related thereto. Keefer and co-workers[5] mentioned the occurrence of fourteen instances of urticaria in over 500 treated patients. Its appearance or disappearance bore no consistent relationship to the administration or withdrawal of the drug. No immunologic investigations had been carried out. Lyons[6] stated that urticaria occurred twelve times in 209 treated patients and was associated with a temperature rise in some. Tests for cutaneous and ophthalmic sensitivity both during and after the active phase of urticaria were negative. The author, however, gave no details about the procedures which were employed. Precipitins were also absent. Dawson and Hobby[7] referred to three instances of urticaria in a group of 100 patients. Herrell, Nichols, and Heilman[8] noted the appearance of "cutaneous sensitivity" in two of 150 treated patients. These authors and others who reported the appearance of urticaria did not conduct any immunologic studies. Pyle and Rattner[9] described the occurrence of contact dermatitis in a medical officer engaged in the preparation of penicillin solutions. A positive patch test with penicillin was demonstrated.

For a number of reasons the group of patients under treatment with penicillin and heparin presented an ideal series on which to study the possible sensitizing effects of penicillin. They were receiving larger doses of the drug than were being administered in most hospitals where penicillin therapy was being studied. Furthermore, they were under treatment for fairly long periods of time. Of greatest significance was the fact that some of them received therapy in interrupted courses. Finally, these patients upon discharge from the hospital were directed to return for clinical checkup, and thus some of them could be tested weeks or months after therapy had been discontinued.

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Received for publication, April 11, 1945.

SKIN TEST STUDIES WITH PENICILLIN SOLUTION

Control Study.—

Technique: The sodium salt of penicillin* was used. This preparation had a potency of approximately 1,000 Oxford units per milligram of the drug. Skin testing was performed by the intracutaneous method. When distilled water was used to dissolve the drug, it was found that the tests with this solution caused a stinging sensation at the injection sites. The use of physiologic saline as the diluent alleviated the discomfort.

The solutions were generally used from one to several hours after their preparation. At times a solution which had been stored in the refrigerator for several days or weeks was employed. Such solutions did not prove irritating nor did they seem to produce smaller reactions than freshly prepared ones.

At the outset a solution consisting of 200 units of penicillin per cubic centimeter was used, but later a concentration of from 5,000 to 10,000 units per cubic centimeter was found to be practical for routine testing. The color of the solution varied from a pale yellow to a light amber with different lots of the salt.

Subjects: When these studies were originally undertaken most of the patients receiving combined therapy were already in various stages of treatment. Several patients, however, were tested before therapy was instituted. Moreover, adequate control studies were made on both nonatopic and atopic subjects. Each control subject was tested with both penicillin solution and with physiologic saline. No ophthalmic tests were performed.

There were at least seventy-five control subjects. These included men and women hospital patients with various ailments, such as coronary artery disease, convalescent pneumonia, arteriosclerotic disease, and others. In addition, there were several patients who were under penicillin therapy for from one to three days. They were regarded as suitable controls because of the early stage of treatment.

The control group included thirty patients under treatment for various allergic complaints. Most of these subjects demonstrated positive reactions to the common allergens.

Results: Each test and saline control were recorded according to the degree of whealing and erythema production. Where indicated, tests were repeated one or several times.

In most instances it was found there was little variation between test and control in regard to the size of the wheal or the extent of erythema formation. The largest wheal was under a 1 plus and the strongest erythema, in or below a 1 plus. It was found that solutions made from some lots of penicillin were more irritating than others, especially those test solutions which were deep amber in color. Retesting of the subjects with solutions made from a more recently obtained lot of the drug frequently elicited essentially negative reactions. These tests thus compared favorably with those done with normal saline. No delayed reactions were found in either the nonatopic or atopic control subjects.

Studies on Patients Receiving Penicillin Treatment.—

Subjects: Twenty-three patients under active therapy were tested from one to six times, the average number of tests for the group being four. Each test was checked with normal saline. Tests were carried out either during the course of therapy or in the intervals between courses. The follow-up tests were done at intervals of weeks or months.

*We are indebted to Mr. John L. Smith, of the Charles Pfizer Co., Brooklyn, N. Y., who supplied the penicillin used in this study.

Seven recovered patients in this group, who returned for a clinical checkup, were tested from twelve to thirty-two weeks after completion of the final course of treatment with penicillin. Also, two patients who were subsequently rehospitalized, one for a reinfection, the other for congestive heart failure, were retested from seven to ten and one-half months after penicillin treatment had been concluded. In addition, four recovered patients, who had never been studied during active treatment, were tested from eight to twenty-two weeks after receiving their last course of penicillin therapy.

Results: The tests were recorded and analyzed as in the control studies. Since these tests were done with newer and, therefore, purer specimens of penicillin, there was even less tendency to produce irritation of the skin than was observed in the control studies. In no instance was there even a wheal or an erythema up to a 1 plus. In most instances the readings compared favorably with those of the saline controls. Where there was a slight increase in skin reactivity at the sites of the penicillin tests, it was considered that this finding fell well within the range of technical variation. No delayed reactions were encountered.

Ophthalmic Tests: One or two drops of the test solution (5,000 Oxford units per cubic centimeter) were instilled into the lower conjunctival sac of one eye. The conjunctiva was watched for at least twenty minutes for evidence of itching, edema, congestion, or lachrymation. There were no positive ophthalmic tests.

Analysis of Cases: An appraisal of such factors as duration of treatment, number of courses given, total dose administered, etc., will emphasize the significance of the findings obtained in this study. Of the twenty-seven patients, fifteen had received one course of treatment, six were given two courses, two had three courses, one had four courses, one was given five courses, and two received six courses. The longest period of treatment in a patient who had been given a single course was twenty-three days. The largest dose administered to a patient receiving a single course of therapy was 4,785,000 Oxford units during a twenty-one-day period. In patients to whom multiple courses were given, the longest course covered a period of fifty-two days, during which 44,500,000 units had been administered. The rest intervals between courses ranged from two to fifty-three days.

The most striking data concern a man who had received six courses of therapy over a total period of 230 days, of which 185 were actual treatment days. The courses ran from twelve to fifty-two days, the free intervals from two to thirteen days. The smallest dose in any one course was 2,700,000 units, and the largest was 44,500,000 units. The total dose given during the entire period of treatment was 112,620,000 Oxford units. This patient did not develop any clinical sensitivity to penicillin nor did he show any positive skin reactions to penicillin.

STUDIES FOR SENSITIVITY TO P. NOTATUM

Test Materials and Procedure.—The solution used for intracutaneous testing was an extract of *P. notatum* equal to .01 or .1 mg. total nitrogen.* All penicillin-treated patients were tested with these solutions and were given a control test with buffered saline. Tests were checked frequently when indicated. As a further control, many of the patients, particularly those who showed question-

*Allergenic extract for skin testing supplied through the courtesy of Dr. A. F. Coca, of Lederle Laboratories, Inc., Pearl River, N. Y.

able or small reactions, were in addition tested with extracts of other molds such as Alternaria, Hormodendrum, *Penicillium rubrum,* and *Penicillium digitatum.*

Results.—In many instances both test and control gave similar findings and many of these were completely negative. In others more whealing and erythema were present at the test site than at the control. Whealing was never larger than a 1 plus. The degree of erythema did not exceed a 1 plus. Furthermore, in all instances where a small reaction was elicited with the extract of *P. notatum,* reactions of similar intensity were brought out by one or usually all of the other molds.

As additional control, in order to determine the average skin reaction produced by testing with *P. notatum,* at least 200 subjects, most of them allergic patients, were tested with this extract and, in most instances, with the other mold extracts mentioned previously. Here again it was found that in the concentration .1 mg. nitrogen per cubic centimeter, the extract of *P. notatum* was essentially nonirritating to the skin and only occasionally produced a small wheal and erythema. In such instances tests with the other mold extracts elicited similar findings.

DISCUSSION

Twenty-seven patients undergoing prolonged intensive therapy with penicillin did not reveal any sensitivity by either intracutaneous or ophthalmic tests. The dosage of penicillin had been high, treatment had been given over long periods of time, and in nearly one-half the patients therapy had been administered in interrupted courses. These circumstances would ordinarily favor active sensitization to the drug employed, if a potent antigenic substance were involved. The results clearly indicate that penicillin is not a potent antigenic product.

Only one report in the literature calls attention to the presence of antibodies to penicillin. Criep[10] demonstrated positive direct and passive transfer tests to penicillin in a patient who had developed urticaria following the first injection in a second course of treatment. Another report of interest immunologically was the demonstration by Welch and Rostenberg[11] of an intense, delayed, tuberculin type reaction resulting from the intracutaneous test with either commercial or crystalline penicillin in a person who had never been treated with the drug. He had been engaged for fifteen years in the handling of a variety of molds. In a footnote these authors state that approximately 5 per cent of 140 unexposed persons exhibited a tuberculin type reaction to the initial injection of crystalline penicillin sodium. This is at variance with the findings obtained in the present study, wherein no delayed reactions were obtained in either atopic or nonatopic control subjects.

The second part of this study revealed the absence of any positive reactions to an extract of the mold *Penicillium notatum.* The counterpart of these results is found in the findings of Feinberg,[12] who studied ten patients who were sensitive both clinically and by scratch test to the molds *P. digitatum* or *P. rubrum.* They gave positive intracutaneous tests with an extract of *P. notatum* but did not react to a solution of penicillin containing 25,000 Oxford units per cubic centimeter.

SUMMARY

Twenty-seven patients receiving intensive and prolonged treatment with penicillin failed to develop any positive skin or ophthalmic reactions to a solu-

tion of penicillin containing 10,000 units per cubic centimeter. These patients did not develop any positive skin reactions to the source of this product, the mold *P. notatum*.

From the present study penicillin would appear to be a very poor sensitizing agent.

REFERENCES

1. Loewe, L., Rosenblatt, P., Greene, H. J., and Russell, M.: Combined Penicillin and Heparin Therapy of Subacute Bacterial Endocarditis, J. A. M. A. **124**: 144, 1944.
2. Loewe, L.: The Combined Use of Penicillin and Heparin in the Treatment of Subacute Bacterial Endocarditis, Canad. M. A. J. **52**: 1, 1945.
3. Loewe, L.: The Combined Use of Anti-infectives and Anticoagulants in the Treatment of Subacute Bacterial Endocarditis, Bull. New York Acad. Med. **21**: 59, 1945
4. Loewe, L., and Rosenblatt, P.: A New Practical Method for Subcutaneous Administration of Heparin, Am. J. M. Sc. **208**: 54, 1944.
5. Keefer, Chester, and others: Penicillin in the Treatment of Infections; Report of 500 Cases; Statement by the Committee on Chemotherapeutic and Other Agents, Division of Medical Sciences, National Research Council, J. A. M. A. **122**: 1217, 1943.
6. Lyons, C.: Penicillin Therapy of Surgical Infections in the U. S. Army, J. A. M. A. **123**: 1007, 1943.
7. Dawson, M. H., and Hobby, G. L.: The Clinical Use of Penicillin: Observation in 100 Cases, J. A. M. A. **124**: 511, 1944.
8. Herrell, W. E., Nichols, D. R., Heilman, D. H.: Penicillin; Its Usefulness, Limitation, Diffusion and Detection, With Analysis of 150 Cases in Which It Was Employed, J. A. M. A. **125**: 1003, 1944.
9. Pyle, H. D., and Rattner, H.: Contact Dermatitis From Penicillin, J. A. M. A. **125**: 903, 1944.
10. Criep, L. H.: Allergy to Penicillin, J. A. M. A. **126**: 429, 1944.
11. Welch, H., and Rostenberg, A., Jr.: Hypersensitivity of the Tuberculin Type to Crystalline Penicillin Sodium, J. A. M. A. **126**: 10, 1944.
12. Feinberg, S. M.: Penicillin Allergy, J. Allergy **15**: 271, 1944.

THE EFFECT OF ARTIFICALLY INDUCED FEVER ON HUMORAL ANTIBODIES AND ON HISTAMINE INTOXICATION IN THE GUINEA PIG

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IN A PREVIOUS report[1] it was demonstrated that sensitivity to horse serum in actively sensitized guinea pigs was suppressed by artificially induced hyperpyrexia. Protection was afforded when sensitized animals were heated to 43.3° C. (110° F.) and immediately injected with horse serum or when they were subjected to a temperature of 42.2° C. (108° F.) for thirty minutes and then given an intravenous injection of antigen. In this connection we have attempted to study the effect of fever on several immunologic reactions to determine, if possible, the mechanism involved in the suppression of anaphylactic shock at febrile temperatures. Since greater protection was obtained in old than in young guinea pigs, we have used, whenever possible, animals 2 to 3½ years of age.

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†From the Kettering Institute for Medical Research, Miami Valley Hospital.
Received for publication, Feb. 16, 1945.

EXPERIMENTAL

The Effect of Fever on the Antiprotein Titer of the Blood Serum.—Ecker and O'Neal[2] and Ellingson and Clark[3] have shown that fever temperatures reduce the antibody titers of rabbits immunized against *Eberthella typhosa*. The effect of fever on humoral antibodies, therefore, was investigated first since quantitative changes in antibody titer might account for a decrease in the sensitivity of an animal toward a specific antigen. For this study seven old male guinea pigs, sensitized by subcutaneous injection of 1.0 c.c. of a 1:100 dilution of horse serum three months previously, were injected intravenously with 0.1 c.c. of a 1:2 dilution of normal horse serum, followed three days later by another injection of 0.1 c.c. of the undiluted antigen. One animal died of anaphylactic shock after the first intravenous injection. The six surviving animals were subjected to high temperature from sixteen to thirty days after the final injection of antigen. One to two hours before, they were bled by incision of a marginal ear vein; they were then placed in the air-conditioned cabinet and a fever temperature of 42.2° C. was induced as previously described.[1] After this rectal temperature had been maintained for sixty minutes, the animals were removed from the cabinet and 0.5 to 1.5 c.c. of blood obtained by incising a vein in the opposite ear. Precipitin tests (antigen-dilution method) were made on the serums using "micro" tubes and graduated capillary pipettes. The results of the titrations are given in Table I. They indicate that fever has no influence on the precipitin titer of the serum, as measured by the antigen dilution method.

TABLE I. THE EFFECT OF A FEVER TEMPERATURE ON THE PRECIPITIN TITER OF IMMUNIZED GUINEA PIGS

PIG	PRECIPITIN TITER BEFORE FEVER TREATMENT	PRECIPITIN TITER AFTER A FEVER TEMPERATURE AT 42.2° C. FOR 60 MINUTES	TIME BETWEEN LAST IMMUNIZING INJECTION AND FEVER SESSION (DAYS)
P 2	1:64	1:64	16
P 3	1:512	1:512	17
P 4	1:64	1:64	20
P 5	0	0	21
P 6	1:64	1:64	27
P 7	1:32	1:32	30

In the following experiment an effort was made to determine the effect of fever on animals whose serum contained only small quantities of antiprotein antibody. This experiment was conducted over a period of four months, and four groups of guinea pigs sensitized at various intervals were used. They were sensitized by subcutaneous injection of 1.0 c.c. of a 1:10 dilution of normal horse serum and were employed from twenty-nine to seventy-six days after sensitization. Before using a group, four to six animals were sacrificed to obtain the approximate minimum lethal dose. As shown in Table II, the lethal dose has varied. The reason for this variation is not clear and indeed in the last group the lethal dose for the individual animals was so variable that an approximate titer could not be established. One-half of the remaining sensitized animals in each group were heated and the others were used for controls. On the day of the heat treatment the animal to be treated was bled. An hour or two later a temperature of 42.2° C. was induced and maintained for sixty minutes. Another bleeding was made and the approximate dose of antigen, as determined previously, was injected intravenously. The control animal was injected on the same day with the same quantity of normal horse serum which was injected into the heat-treated animal.

TABLE II. THE EFFECT OF FEVER ON THE SERUM ANTIPROTEIN TITER OF OLD GUINEA PIGS SENSITIZED TO HORSE SERUM

PIG	HEAT TREATED ANIMALS								CONTROL ANIMALS		
	TITRATION OF SERUMS										
	SERUM DILUTION	ACCEPTOR ANIMALS		MEAN DIAMETERS OF REACTIONS*		RATIO	DOSE OF HORSE SERUM INJECTED (C.C.)	RESULTS	PIG	DOSE OF HORSE SERUM INJECTED (C.C.)	RESULTS
		NO. INJECTED	NO. POSITIVE	SERUM OBTAINED BEFORE FEVER	SERUM OBTAINED DURING FEVER						
Group 1											
JJ 511	1:4	4	4	13.3 by 16.3	12.0 by 15.0	0.83	0.75	Slight shock; recovered	S 261	0.75	Died; 3 min.
T 574	1:4	3	3	15.0 by 18.6	14.0 by 16.3	0.82	-†	-	-	-	-
Group 2											
C 963	1:4	6	6	19.6 by 21.6	16.5 by 20.0	0.78	0.75	Slight shock; recovered	S 197	0.75	Died; 6 min.
A 661	1:4	12	3	14.0 by 17.3	13.6 by 17.3	0.97	0.75	Slight shock; recovered	S 847	0.75	Died; 4 min.
JJ 249	1:4	6	0	0	0		0.75	No symptoms	C 477	0.75	Moderate shock; recovered
Group 3											
B 205	1:4	6	5	15.0 by 18.7	14.8 by 18.7	0.99	0.875	Slight shock; recovered	O 931	0.875	Moderate shock; recovered
B 214	1:4	6	4	21.7 by 23.5	20.0 by 22.0	0.87	0.875	Slight shock; recovered	C 571	0.875	Died; 5 min.
A 627	1:4	6	5	23.0 by 25.0	23.0 by 26.0	1.04	0.875	Slight shock; recovered	C 522	0.875	Died; 5 min.
A 628	1:4	6	5	26.4 by 28.4	26.8 by 28.2	1.01	1.0	Moderate shock; recovered	B 223	1.0	Died; 5 min.
	1:8	6	5	18.4 by 20.0	18.4 by 19.2	0.96					
Group 4											
O 34	1:4	6	1	11.0 by 13.0	13.0 by 14.0	1.27	-		-		
D 305	1:4	6	3	16.6 by 20.3	17.6 by 19.6	1.02	-		-		
DD 658	1:4	6	6	16.0 by 18.7	17.2 by 19.5	1.12	-		-		
J 693	1:4	6	6	12.8 by 17.0	13.8 by 15.0	0.96	-		-		

*Diameter of reactions in millimeters.
†Not done.

In Table II it is shown that all of the heat-treated animals injected with a dose of horse serum which was lethal for six of the eight control animals recovered. Six of these showed slight shock and one, moderate shock. One animal, JJ 249, was probably not sensitive since symptoms of anaphylaxis were not evident after injection of an assaulting dose of horse serum, and its serum did not confer passive sensitivity to the skin of recipient animals.

The antihorse serum antibody content of the sera from the heat-treated animals was titrated by passive transfer of sensitivity to the skin of guinea pigs weighing 300 grams. The size of the reaction following an intraperitoneal injection of antigen was taken as an index of the antihorse serum antibody content of the serum (Prausnitz-Küstner reaction). The serum obtained before induction of fever was usually diluted with three parts of saline and 0.05 c.c. was injected intradermally into three to six guinea pigs weighing 285 to 310 grams. The injections were made on one side of the median line of the back, midway betwen scapula and flank. The serum obtained at the end of the fever period was also diluted 1:4 and injected intradermally at a corresponding site of the opposite side of the back. Only two injections were made into each animal. The location of the two sera on the recipient animals was alternated so that the serum under test was treated as an unknown and measurements of the reactions on the following day were made without knowledge of which antiserum was injected. From eighteen to twenty-four hours after the intradermal injections, 0.5 c.c. of normal horse serum was administered intraperitoneally, as recently suggested by Chase.[4] Intraperitoneal injection was found to be superior to local injection of antigen at the sensitized site because the reactions were not complicated by the wheal formed by the intradermal injection of horse serum.

Not all of the recipients reacted after intraperitoneal injection of antigen. The wheals or areas of erythema were measured at the end of fifteen, thirty, forty-five, sixty, and ninety minutes. In the majority of the animals the reactions were at their height sixty minutes after administration of antigen, and these measurements were used as a basis for comparing the potency of the serums. Those animals in which either one or both reactions did not have well-defined margins were not included in the calculations. In reading the reactions two diameters at right angles to each other were measured and the mean dimensions obtained. The ratio was calculated from the products of these mean measurements. The number of recipient animals, the mean diameters, and the ratios are given in Table II. The data show that there is no significant difference in the titer of the serum obtained before fever and that obtained after sixty minutes of fever at 42.2° C.

Tests were made to determine whether this passive transfer method of titration could differentiate between two sera having different potencies. A pooled serum was used which was obtained from ten young guinea pigs sensitized to normal horse serum thirty-six days previously. It was diluted 1:10 instead of 1:4 because on preliminary tests it appeared to be stronger than the sera from the old guinea pigs used in the experiment just described. Two dilutions were injected, a 1:10 dilution and a 1:16.6 dilution, or a difference in potency of 40 per cent. The location of the antisera on the backs of the animals was alternated as previously described. The results given in Table III show that a difference in potency of 40 per cent is easily distinguishable, the sixty-minute readings giving a ratio of 0.61. This titration was controlled by injecting a 1:10 dilution of antiprotein serum on both sides of the backs of young guinea pigs. The measurements made sixty minutes after injecting 0.5 c.c. of normal horse serum

TABLE III. A COMPARISON OF THE SKIN REACTIONS ELICITED BY INTRAPERITONEAL INJECTION OF HORSE SERUM IN GUINEA PIGS PREVIOUSLY SENSITIZED LOCALLY WITH A 1:10 AND A 1:16.6 DILUTION OF ANTIHORSE SERUM ANTIBODY

PIG	WEIGHT (GM.)	DILUTION	READING*	DILUTION	READING*
F 195	305	1:16.6	0	1:10	0
F 193	290	1:16.6	0	1:10	0
F 144	315	1:16.6	9 by 13 by 0.5	1:10	11 by 16 by 1.0
F 157	300	1:16.6	10 by 12 by 0.5	1:10	13 by 16 by 1.0
F 182	315	1:16.6	9 by 11†	1:10	11 by 14†
J 148	290	1:16.6	11 by 14 by 0.5	1:10	12 by 15 by 0.5
J 125	300	1:16.6	10 by 13 by 0.5	1:10	14 by 18 by 0.5
J 135	305	1:16.6	17 by 19 by 1.0	1:10	21 by 23 by 2.0
J 3	295	1:16.6	13 by 15 by 0.5	1:10	17 by 20 by 1.0
J 106	315	1:16.6	0	1:10	0
J 1	315	1:16.6	0	1:10	0
AA 809	300	1:16.6	6 by 6†	1:10	9 by 10†
AA 919	295	1:16.6	12 by 14†	1:10	14 by 15†
CC 956	280	1:16.6	0	1:10	0
AA 936	300	1:16.6	9 by 14†	1:10	12 by 14†
AA 406	300	1:16.6	7 by 8†	1:10	10 by 14†
CC 934	290	1:16.6	7 by 8†	1:10	11 by 12†
Mean diameters of reactions			10.0 by 12.2		12.9 by 15.6

Ratio of areas: 0.61.
*Diameter and elevation of reactions in millimeters.
†Erythematous reaction; no edema.

TABLE IV. A COMPARISON OF THE SKIN REACTIONS ELICITED BY INTRAPERITONEAL INJECTION OF HORSE SERUM IN GUINEA PIGS PREVIOUSLY SENSITIZED LOCALLY WITH TWO INJECTIONS OF A 1:10 DILUTION OF ANTIHORSE SERUM ANTIBODY

PIG	WEIGHT (GM.)	DILUTION	READING*	DILUTION	READING*
J 156	290	1:10	10 by 15†	1:10	9 by 14†
E 844	290	1:10	16 by 20†	1:10	16 by 20†
J 154	295	1:10	11 by 17†	1:10	12 by 17†
J 183	290	1:10	17 by 22 by 1.0	1:10	16 by 22 by 1.0
F 17	300	1:10	0	1:10	0
J 519	290	1:10	0	1:10	0
F 44	290	1:10	14 by 18 by 1.0	1:10	14 by 19 by 1.0
J 44	300	1:10	17 by 21 by 2.0	1:10	16 by 21 by 2.0
J 2	310	1:10	13 by 16 by 1.0	1:10	13 by 15 by 1.0
J 79	285	1:10	13 by 19 by 0.5	1:10	14 by 19 by 0.5
H 589	285	1:10	0	1:10	0
CC 629	310	1:10	11 by 13†	1:10	11 by 15†
AA 731	310	1:10	10 by 14†	1:10	11 by 14†
AA 789	310	1:10	0	1:10	0
AA 795	310	1:10	13 by 16†	1:10	12 by 16†
CC 708	285	1:10	0	1:10	0
Mean diameters of reactions			13.2 by 17.4		13.1 by 17.5

Ratio of areas: 1.0.
*Diameter and elevation of reactions in millimeters.
†Erythematous reaction; no edema.

intraperitoneally are given in Table IV. The ratio obtained was 1.00, indicating that the method can be used for quantitative determination of the antiprotein titer of serums.

One-quarter cubic centimeter syringes and short beveled 27-gauge needles were used for making the intradermal injections. It was found that leakage of the diluted antiprotein serum after intradermal injection could be prevented by keeping the needle in place in the skin for from six to ten seconds after injection. Young guinea pigs were used for titration because old ones injected with the same or with more concentrated solutions of antiprotein serum intradermally did not react, even though the amount of antigen injected intraperitoneally, calculated from the weight of the animals, was proportional to that given to the young guinea pigs.

The Effect of Fever on Histamine Intoxication.—Since a febrile temperature did not appear to alter the humoral antibody content of the serum significantly but did reduce sensitivity toward the specific antigen, the effect of fever on a nonspecific stimulant, histamine, was studied. Histamine was chosen because practically all of the symptoms of anaphylactic shock are thought to be due to liberation of this substance during the antigen-antibody reaction.[5] Twenty old guinea pigs were kept in the heat cabinet at 42.2° C. for thirty minutes, and at the end of this period a dose of histamine dihydrochloride* was injected intravenously. The dose of histamine which killed 80 to 100 per cent of the control animals was determined by preliminary titration on guinea pigs of approximately the same age and weight. As a control, one nonheat-treated guinea pig for each treated animal was injected at the same time and with the same quantity of histamine. The histamine solutions were freshly prepared each day and the concentrations reported below refer to the free base. The results given in Table V show that thirteen of the twenty heat-treated animals recovered, whereas only one of the control animals survived. For the male animals, the dose of histamine was again determined by preliminary titration on a small group. From these results it appeared that they were more susceptible to histamine and that 0.18 mg. was sufficient to kill them. Unfortunately, at that time a large number of male animals was not available to determine whether this was the case. When old boars were again available, a comparison of the toxicity of histamine in old male and female guinea pigs was made. The results, which are not given in detail, indicated that there was no essential difference in the toxicity of histamine in the two sexes.

TABLE V. THE EFFECT OF A FEVER TEMPERATURE OF 42.2° C. MAINTAINED FOR THIRTY MINUTES ON THE RESPONSE TO HISTAMINE IN OLD GUINEA PIGS

FEVERED ANIMALS				CONTROL ANIMALS			
PIG	DOSE OF HISTAMINE* (MG.)	RESULTS	SEX	PIG	DOSE OF HISTAMINE* (MG.)	RESULTS	SEX
H 5	0.24	Died; 4 min.	F	Q 968	0.24	Died; 2 min.	F
H 6	0.24	Recovered	F	I 137	0.24	Died; 2 min.	F
A 617	0.24	Died; 6 min.	F	C 908	0.24	Died; 5 min.	F
A 697	0.24	Recovered	F	H 7	0.24	Died; 5 min.	F
H 8	0.24	Recovered	F	C 968	0.24	Died; 2 min.	F
A 674	0.24	Recovered	F	C 511	0.24	Died; 2 min.	F
H 10	0.24	Recovered	F	H 9	0.24	Died; 2 min.	F
C 980	0.24	Died; 2 min.	F	R 727	0.24	Died; 3 min.	F
H 65	0.24	Recovered	F	R 465	0.24	Died; 2 min.	F
BB 519	0.24	Recovered	F	A 647	0.24	Recovered	F
A 662	0.24	Died; 3 min.	F	U 620	0.24	Died; 2 min.	F
B 982	0.24	Recovered	F	JJ 824	0.24	Died; 2 min.	F
D 400	0.24	Recovered	F	W 922	0.24	Died; 2 min.	F
H 10	0.24	Died; 5 min.	F	H 11	0.24	Died; 60 min.	F
H 12	0.24	Recovered	F	A 697	0.24	Died; 3 min.	F
Q 765	0.24	Recovered	M	N 11	0.24	Died; 2 min.	F
H 13	0.21	Recovered	M	B 541	0.18	Died; 2 min.	M
H 14	0.18	Died; 60 min.	M	J 640	0.18	Died; 2 min.	M
H 15	0.18	Recovered	M	W 679	0.18	Died; 3 min.	M
H 17	0.18	Died; 2 min.	M	H 16	0.18	Died; 3 min.	M
Recovered		13				1	
Died		7				19	

*Calculated as the free base.

To ascertain whether guinea pigs were protected after heat treatment and after their temperatures were allowed to return to normal, the following experiment was made. Ten old female guinea pigs were kept at a temperature of 42.2° C. for thirty minutes. At the end of this period the cabinet doors were opened and defervescence allowed to take place. After the animals attained their

*Eastman Kodak Co., Rochester, N. Y.

prefever temperature, a dose of histamine was injected intravenously. For a control, one nontreated animal was injected at the same time and with the same dose of histamine. The results shown in Table VI indicate that the animals are protected against histamine only during the height of fever. After the temperature has returned to normal, they are as susceptible to the stimulant as are the control animals.

TABLE VI. THE EFFECT OF A FEVER TEMPERATURE OF 42.2° C. MAINTAINED FOR THIRTY MINUTES ON THE RESPONSE TO HISTAMINE WHEN INJECTED AFTER THE TEMPERATURE HAD RETURNED TO NORMAL

FEVERED ANIMALS			CONTROL ANIMALS		
PIG	DOSE OF HISTAMINE* (MG.)	RESULTS	PIG	DOSE OF HISTAMINE* (MG.)	RESULTS
C 914	0.24	Died; 1 min.	A 607	0.24	Died; 3 min.
CC 704	0.24	Died; 2 min.	BB 339	0.24	Died; 2 min.
D 235	0.24	Died; 3 min.	N 1	0.24	Died; 4 min.
JJ 880	0.24	Died; 3 min.	W 767	0.24	Died; 3 min.
JJ 871	0.24	Recovered	D 268	0.24	Died; 30 min.
HH 317	0.24	Died; 3 min.	N 2	0.24	Died; 4 min.
N 550	0.24	Died; 2 min.	N 3	0.24	Died; 45 min.
H 624	0.24	Died; 16 min.	R 739	0.24	Died; 3 min.
I 534	0.24	Died; 3 min.	B 315	0.24	Died; 3 min.
Q 317	0.24	Died; 4 min.	Q 930	0.24	Recovered
Recovered		1			1
Died		9			9

*Calculated as the free base.

The Response of Intestinal Strips Toward Histamine When Exposed at Various Temperatures.—The effect of histamine on surviving intestinal strips was studied to determine whether the same temperature shown to suppress anaphylactic shock in vivo influenced the degree of contraction of this tissue. In these experiments the tissue was heated to a temperature of 43.3° C. (110° F.) since it was demonstrated that bringing sensitized guinea pigs to this temperature afforded protection against anaphylactic shock. Old normal female guinea pigs were used. They were sacrificed and as soon as reflex movement ceased, the abdomen was opened and the ileum removed and placed in warm Ringer-Locke's solution. The ileum was carefully dissected from the mesentery and cut into segments approximately 12 cm. in length. Detritus within the lumen was washed out by allowing large quantities of warm Ringer-Locke's solution to flow through by gravity. The segments were placed in separate beakers containing nutrient solution and were used immediately or stored at 5° C. until needed. Nicoll and Campbell[6] found that at a temperature of 5° C. ileal segments retain their activity for from three to five days. We have verified this observation. Before use, the 12 cm. segments of ileum were cut into four strips approximately 3 cm. in length and one or two of these strips suspended in a tissue bath containing Ringer-Locke's solution. Oxygen was bubbled through the tissue bath throughout the experiment. The tissue bath was suspended in a constant temperature bath regulated at 38.8 or 39.0° C. (101.8 or 102.2° F.). This temperature was 0.3 and 0.05° C., respectively, lower than the mean rectal temperature of the heat-treated guinea pigs injected with histamine. The tissues were connected to muscle levers with silk thread and recordings made as described by Dale.[7] Instead of smoked paper, the ink writing pointers described by Friedman[8] were employed. The tissue was allowed to remain in the oxygenated nutrient solution for from forty to fifty minutes before being tested with histamine. The quantity of histamine added to the muscle bath was an amount sufficient to provoke a submaximal contraction of the tissue. The tis-

sue was washed with three changes of nutrient solution having the same temperature as the bath. The temperature was then increased to 43.3° C. This usually required from forty to fifty minutes. When this temperature was attained, the same quantity of histamine was added. After washing the tissue three times, the bath was cooled to 38.8° C. by running cold water through a copper coil suspended in the bath. The same amount of histamine was then added as before. Three typical tracings are shown in Fig. 1. They indicate that there is less contraction of the tissue at 43.3° C. than at 38.8 or 39.0° C. Thirty-five of these experiments were made with tissue derived from six old guinea pigs. All except two of the tissues showed less response at 43.3° C. than at 38.8 or 39.0° C. when exposed to the same concentration of histamine. The average magnitude of the contractions after the first addition of histamine at 38.8 or 39.0° C. was 13.1 mm., at 43.3° C. it was 7.0 mm., and after the last addition at 38.8 to 39.0° C. it was 14.4 mm.

In other experiments the temperature was raised to 45° C. (113° F.) or 46.0° C. (114.8° F.) and the tissue exposed to histamine. Two typical tracings are shown in Fig. 2. They indicate that there is a marked decrease in response

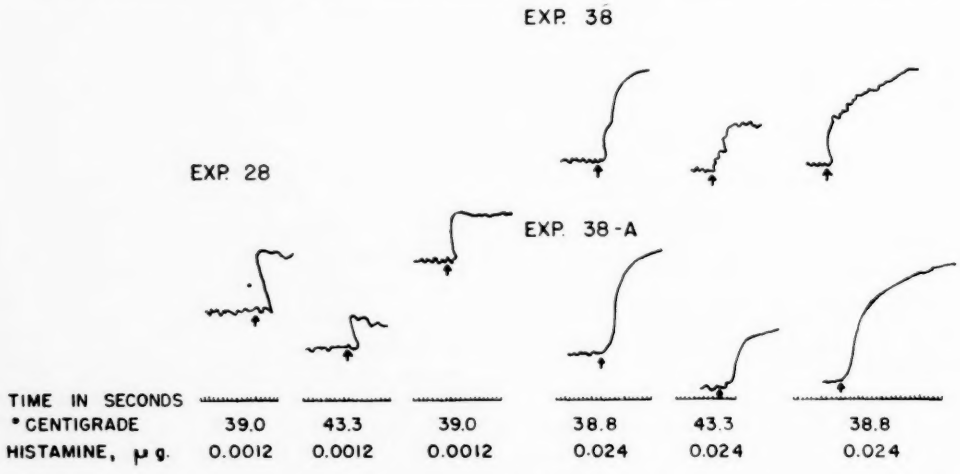


Fig. 1.—The effect of temperatures of 38.8 to 39.0° C. and 43.3° C. on the response of ileal strips to histamine.

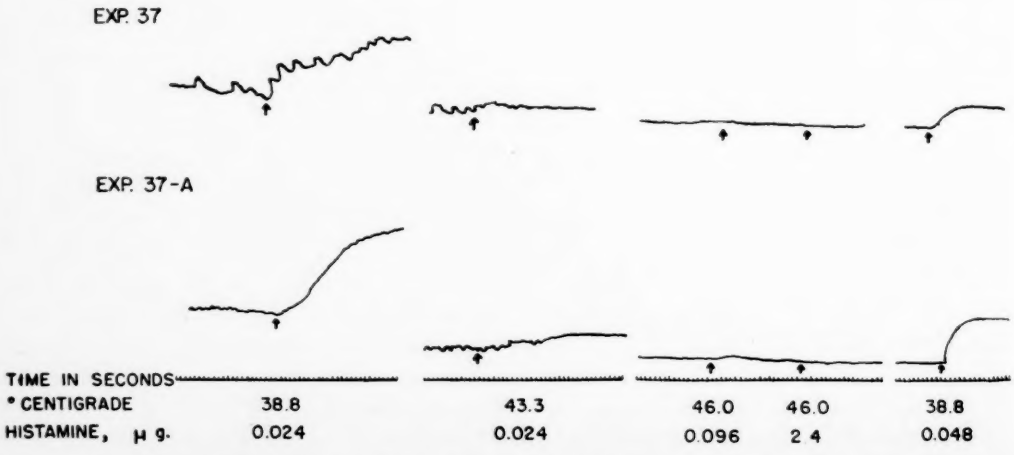


Fig. 2.—The response of ileal strips to histamine at 38.8, 43.3, and 46.0° C.

at these temperatures. The maximum amount of the stimulant to which the tissues were exposed at these elevated temperatures was 2.4 μg per cubic centimeter. As shown in Fig. 2, there is less contraction to 2.4 μg per cubic centimeter of histamine at 46.0° C. than to 0.024 μg at 38.8° C.

The Response of Sensitized Intestinal Strips Toward the Specific Antigen When Exposed at Various Temperatures.—Experiments were made with tissue from animals sensitized by intraperitoneal injection of 100 mg. of ovalbumin. Animals weighing approximately 500 grams were used. We were unable to sensitize old guinea pigs to such an extent that a response of the excised ileum to antigen could be provoked consistently when the animals received a single sensitizing dose of ovalbumin. The animals were used from twenty-nine to eighty-six days after the sensitizing injection had been given. The ovalbumin was recrystallized four times according to the method of Cole.[9] Two strips from a 12 cm. segment of ileum were set up in the tissue bath at 38.8 or 39.° C. and the temperature was slowly raised to 43.3° C. (from forty to fifty minutes). An amount of ovalbumin was added which, on preliminary test with a trial strip of intestine from the same animal, gave a submaximal contraction. For a con-

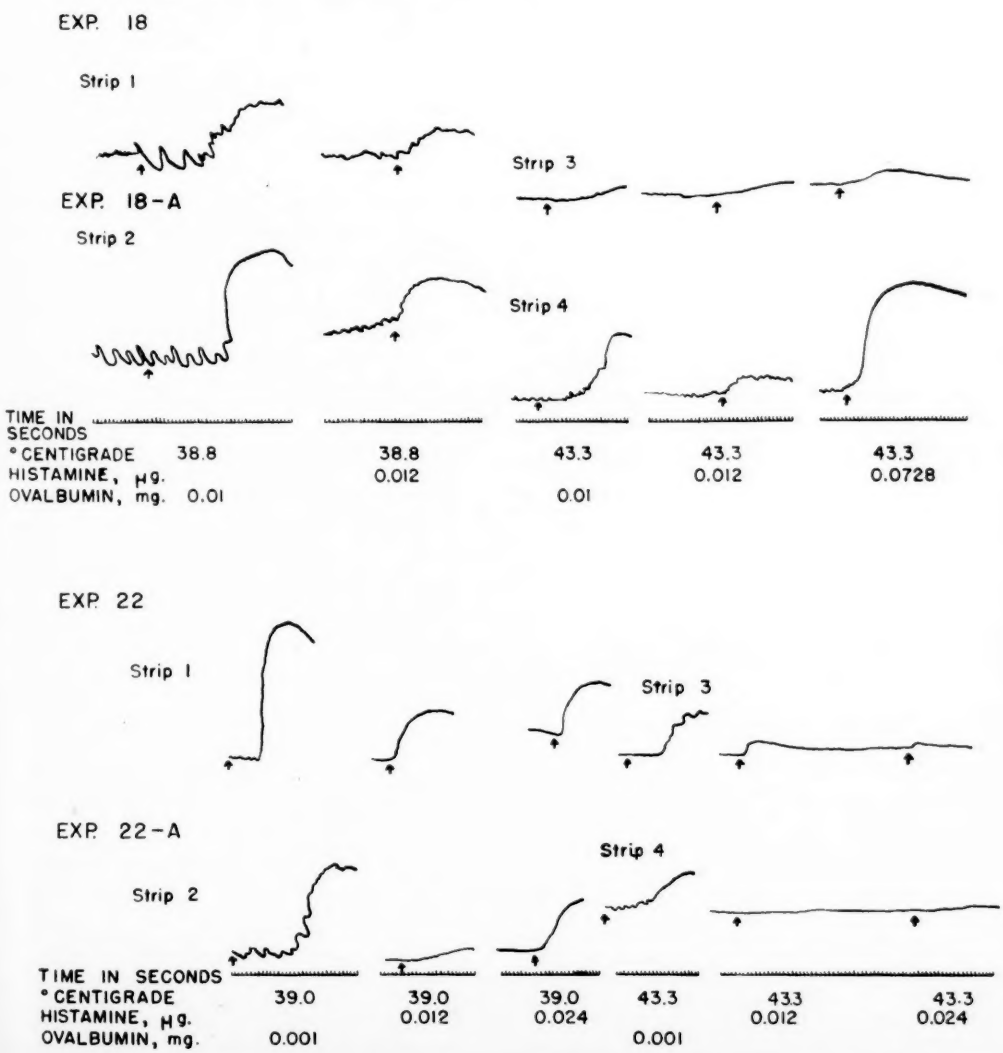
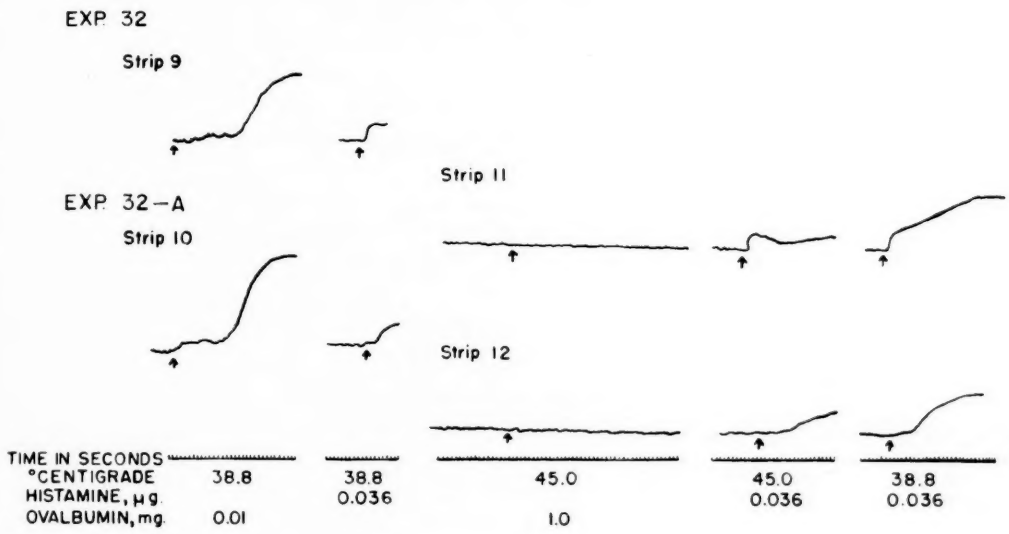


Fig. 3.—The response of sensitized ileal strips to ovalbumin and histamine when exposed to these substances at 38.8 to 39.0° C. and at 43.3° C.

trol the two remaining strips were kept at 38.8 or 39.0° C. for from forty to fifty minutes. These tissues were exposed to the same amount of ovalbumin as the heated segments. Forty-six of these experiments were made with tissue from nine guinea pigs. In thirty-eight of the tests there was less contraction in the strips heated to 43.3° C. than in the control strips kept at 38.8 or 39.0° C. for from forty to fifty minutes. Eight strips showed equal or greater contraction at the elevated temperature. Four typical tracings are shown in Fig. 3. The mean magnitude of the contractions of the control strips was 17.5 mm. and for the heated strips it was 10.0 mm.

Strips were also heated to 45.0 or 46.0° C. In none of these tests was there any contraction, even when the tissue was exposed to 1.0 mg. per cubic centimeter of antigen. Several typical tracings are shown in Fig. 4. Experiments were then made to determine whether histamine-like substances were released from sensitized ileum at these temperatures. For these tests guinea pigs weighing from 390 to 590 grams were sensitized by a single intraperitoneal injection of 100 mg. of crystallized ovalbumin. From thirteen to thirty-three days later they were killed and approximately 40 cm. of the ileum removed. Detritus within the lumen was washed out with warm Ringer-Locke's solution and the tissue was cut into segments 10 cm. in length. The segments were closed with silk thread. One segment was placed in a culture tube containing 1.0 c.c. of Ringer-Locke's solution previously immersed in a constant temperature bath regulated at 39.0° C. The adjacent segment was also placed in a culture tube containing 1.0 c.c. of Ringer-Locke's solution, but it was immersed in a constant temperature bath regulated at 45.0° C. Three minutes later, after the muscle had attained the temperature of the bath, 1.0 or 10.0 mg. of ovalbumin contained in 1.0 c.c. of Ringer-Locke's solution and having the same temperature as the bath was added to each tissue. The tissues were agitated several times and five minutes after addition of antigen, 1.0 c.c. of liquid was removed from each tube and tested on the excised ileum from normal guinea pigs weighing 300 grams. The strip of normal ileum was suspended in a tissue bath regulated at 39.0° C. which contained 10.0 c.c. of Ringer-Locke's solution. Histamine-like substances could not be detected in all of the tissues tested. Several tracings are shown in Fig. 5. They show that histamine-like substances are released from sensitized ileum at 45.0° C. We were unable to compare accurately the amounts released at 39.0 and 45.0° C. because of the variation in the quantity released by different strips of tissue. Since histamine-like substances are released at 45.0° C., however, it is probable that the effect of heat on sensitized tissue is primarily due to a decrease in the sensitivity of smooth muscle toward the toxic substance released and not to a decrease in the reaction between antigen and fixed antibody.

The Effect of Fever on the Immediate Type of Skin Reaction.—Our next concern was whether fever temperatures decreased the sensitivity of sensitized skin tissue as well as of smooth muscle tissue. The size of the wheal in the immediate type of reaction during fever was studied. For these tests guinea pigs weighing approximately 600 grams were used. The reactions produced in this size animal are not as large as the reactions elicited in guinea pigs weighing from 250 to 300 grams, but because such animals are less excitable and therefore easier to handle in the heat cabinet than young animals, they were used. The day before the heat treatment three or four closely clipped animals were injected in the following manner: On both sides of the back and at identical sites, 0.05 c.c. of undiluted antiprotein serum was injected intradermally. The antiprotein serum



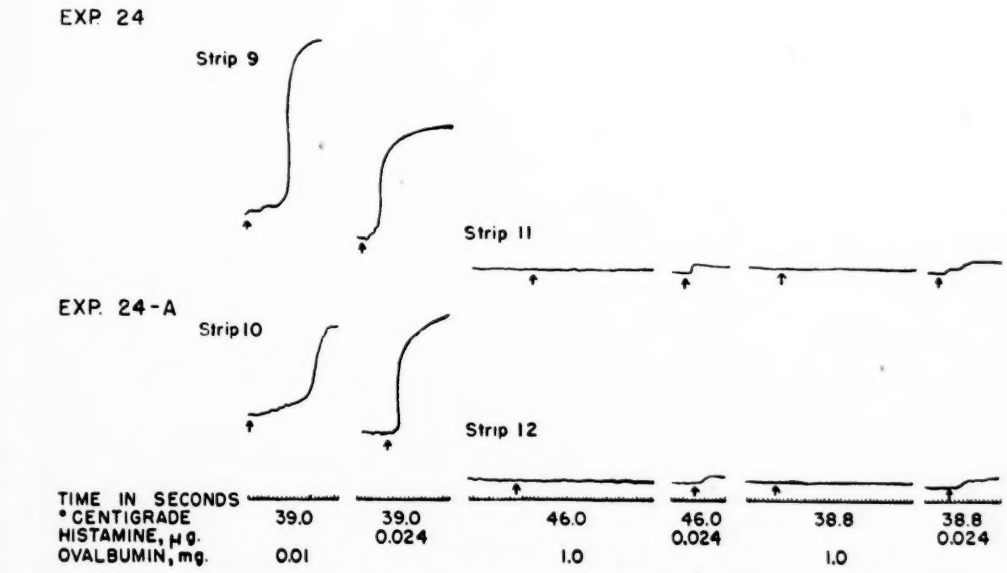


Fig. 4.—The effect of temperatures of 38.8 to 39.0, 45.0, and 46.0° C. on the response to ovalbumin and histamine in sensitized ileal strips.

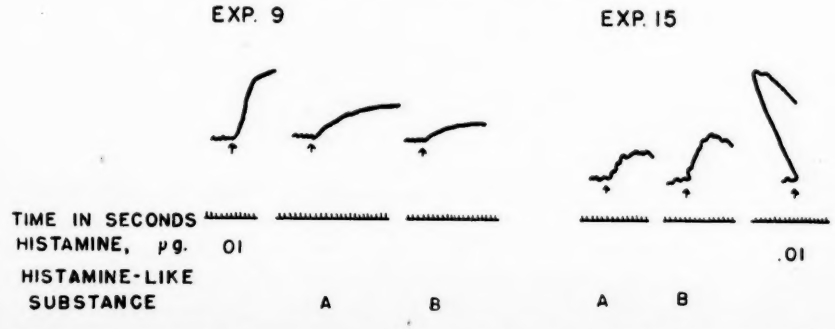


Fig. 5.—The effect of a temperature of 45.0° C. on the release of histamine-like substances from sensitized guinea pig ileum after exposure to antigen. *A*, Sensitized ileum and 0.5 mg. of ovalbumin per cubic centimeter incubated at 38.9° C. *B*, Sensitized ileum and 0.5 mg. of ovalbumin per cubic centimeter incubated at 45.0° C.

was a pooled serum obtained from young guinea pigs sensitized to horse serum. For a control, 0.05 c.c. of undiluted normal guinea pig serum was injected approximately 3 cm. anterior to, and on either one or the other side of, the back. On the following day 0.05 c.c. of normal horse serum was injected intradermally into one sensitized site and into the control site previously injected with normal guinea pig serum. Sixty minutes later the reactions were measured. The animals which did not react to the injection of normal horse serum at the sensitized site were discarded. One of the animals which showed an immediate type of response to the injection of normal horse serum at the sensitized site was heated at 42.2° C. and the temperature maintained for thirty minutes. The animal was then removed and 0.05 c.c. of normal horse serum was injected into the remaining sensitized site. It was returned to the fever cabinet and the temperature of 42.2° C. maintained for thirty minutes. At the end of this time the cabinet doors were opened and defervescence was allowed to take place. Thirty minutes later, or sixty minutes after injection of antigen, the reaction was measured in two diameters. At approximately the same time that the heat-treated animal was injected with normal horse serum, the remaining control animals that reacted to the antigen were given the same dose of horse serum at the remaining sensitized site. Sixty minutes after injection these reactions were likewise measured in two diameters. The results of these tests are given in Table VII. They show that the wheals formed during hyperpyrexia are significantly smaller than those formed before induction of fever. It is apparent from these results that skin as well as smooth muscle tissue is desensitized during hyperpyrexia.

TABLE VII. COMPARISON OF THE IMMEDIATE TYPE REACTIONS ELICITED BY INTRADERMAL INJECTION OF NORMAL HORSE SERUM IN THE SKIN OF LOCALLY SENSITIZED GUINEA PIGS BEFORE AND DURING A FEVER TEMPERATURE OF 42.2° C.

PIG	FEVERED ANIMALS — SIZE OF WHEALS ELICITED BY HORSE SERUM IN MM.			PIG	UNFEVERED CONTROL ANIMALS — SIZE OF WHEALS ELICITED BY HORSE SERUM IN MM.		
	(SENSITIZED SITE) INJECTED BEFORE FEVER	(SENSITIZED SITE) INJECTED 3 TO 4 HOURS AFTER (1) DURING FEVER	(NORMAL SITE) INJECTED BEFORE FEVER		(SENSITIZED SITE)	(SENSITIZED SITE) INJECTED 3 TO 4 HOURS AFTER (1)	(NORMAL SITE)
	(1)	(2)	(3)		(1)	(2)	(3)
C 753	18 by 23	16 by 19	14 by 18	I 643	19 by 21	20 by 22	16 by 20
M 723	24 by 25	16 by 18	19 by 22	I 633	15 by 21	15 by 19	14 by 15
I 680	15 by 18	13 by 15	12 by 12	D 319	16 by 20	21 by 21	13 by 18
J 954	19 by 21	14 by 17	15 by 15	I 171	20 by 22	22 by 23	15 by 17
D 722	17 by 17	12 by 13	14 by 15	I 604	21 by 25	20 by 23	15 by 15
I 466	18 by 20	16 by 19	11 by 16	I 673	20 by 24	20 by 24	13 by 16
DD 573	16 by 21	14 by 17	14 by 16	R 109	15 by 19	15 by 19	14 by 16
V 292	18 by 20	14 by 16	14 by 17	A 749	14 by 18	14 by 18	11 by 16
DD 505	16 by 17	12 by 15	12 by 14	K 736	16 by 18	15 by 18	15 by 16
DD 512	17 by 22	16 by 19	13 by 15	V 282	17 by 19	17 by 19	16 by 17
				V 240	16 by 17	17 by 19	12 by 14
				V 233	15 by 15	17 by 19	12 by 15
				DD 574	16 by 20	17 by 19	15 by 15
				DD 599	13 by 17	18 by 22	11 by 13
Mean diameters	17.8 by 20.4	14.3 by 16.8	13.8 by 16.0		16.6 by 19.7	17.7 by 20.4	13.7 by 15.9

DISCUSSION

The foregoing experiments indicate that although there is no significant change in the antiprotein titer of an animal's serum during hyperpyrexia, sensitivity to the specific antigen is decreased. All of the heat-treated animals injected with antigen recovered, while six of the eight control animals given the same dose of horse serum died of anaphylactic shock. Since febrile temperatures

had no effect upon the antiprotein content of the serum but did suppress anaphylactic shock, the effect of fever on histamine intoxication was studied to ascertain whether hyperpyrexia affected sensitivity toward this nonspecific stimulant. The results show that resistance to histamine shock is increased at fever temperatures since 95 per cent of the control animals and only 33 per cent of the heat-treated animals died when given the same dose of histamine. Protection against histamine shock is obtained only during the height of fever. When the temperature has returned to normal after a temperature of 42.2° C. has been maintained for thirty minutes, the animals are as susceptible to histamine as the nonheat-treated control animals. Mirsky and Wasserman[10] also studied the effect of fever on histamine in a series of guinea pigs. They found, however, that fever had no effect on histamine intoxication.

The protection afforded by a fever temperature against histamine is not as great as that afforded against anaphylactic shock. Thirty-five old sensitized guinea pigs, used in these and other experiments not reported here, were heat-treated at a temperature of 41.7 or 42.2° C. for periods of thirty or sixty minutes. At the end of the fever period a dose of horse serum was injected. Eighty-eight per cent of the control animals died, while only 3 per cent of the heat-treated animals given the same dose of antigen succumbed. Although it was found difficult to compare the degree of severity in histamine shock with the degree of severity in anaphylactic shock, the symptoms in old heat-treated animals injected with histamine appeared to be more severe than the symptoms in old similarly treated sensitized animals injected with horse serum.

The in vitro studies on the surviving intestine exposed to histamine make it appear probable that a decrease in contractility of this tissue during fever is responsible for the protection afforded against this stimulant in vivo. The results show that as the temperature is increased from 38.8 to 46.0° C., contractility of excised smooth muscle, when exposed to histamine, decreases. When the same tissue was tested at 38.8° C., at 43.3° C., and again, after cooling, at 38.8° C., approximately the same response was obtained after the last as after the first exposure to histamine. This appears to indicate that there is little tissue damage at 43.3° C. This was not the case after exposure to temperatures of 45 or 46° C., however, since there was always less response to histamine after the tissue was cooled at 38.8° C.

In thirty-eight of forty-six tests, intestinal strips from animals sensitized to ovalbumin likewise showed less response at 43.3° C. than at 38.8° C. The eight failures may have been due to the fact that ileal strips from the same animal are not exactly identical in their sensitivity. Slight differences in the length of the tissue, for which we did not attempt to correct, may also play a role. In the experiments conducted at 45 and 46° C. there was marked loss of response on exposure to ovalbumin. These in vitro experiments indicate that a decrease in contractility of smooth muscle tissue at elevated temperatures may be partially responsible for the protection afforded by fever against anaphylactic shock in vivo. The greater protection afforded by fever to sensitized guinea pigs injected with an assaulting dose of antigen than to guinea pigs injected with histamine makes it appear probable that still other factors play a role in the desensitization of sensitized guinea pigs by fever temperatures.

SUMMARY

An artificially induced fever temperature of 42.2° C. maintained for thirty minutes has no effect upon the humoral precipitin titer of guinea pigs immunized against horse serum.

The antiprotein titer of sensitized guinea pigs is not significantly altered by a fever temperature of 42.2° C. maintained for sixty minutes when the serum is titrated by a modified Prausnitz-Küstner reaction.

A fever temperature of 42.2° C. maintained for thirty minutes suppresses histamine shock.

In vitro tests with surviving normal intestine exposed to histamine indicate that there is less contraction at 43.3, 45.0, or 46.0° C. than at 38.8 or 39.0° C. Response of sensitized intestine to the specific antigen (ovalbumin) is also decreased by temperatures of 43.3, 45.0, or 46.0° C.

The immediate type of reaction in the guinea pig's skin is suppressed by a fever temperature of 42.2° C. maintained for sixty minutes when the locally sensitized tissue is injected with the specific antigen during hyperpyrexia.

REFERENCES

1. Gottschall, R. Y., deKruif, P., Cope, H. E., and Laurent, D.: The Effect of Artificially Induced Fever on Anaphylactic Shock in Actively Sensitized Guinea Pigs, J. Lab. & Clin. Med. **29**: 614, 1944.
2. Ecker, E. E., and O'Neal, M.: Effect of Hyperpyrexia Induced by an Ultra-high Frequency Current on B. Typhosus Agglutinin and Complement, Am. J. Pub. Health **22**: 1050, 1932.
3. Ellingson, H. V., and Clark, P. F.: The Influence of Artificial Fever on Mechanisms of Resistance, J. Immunol. **43**: 65, 1942.
4. Chase, M. W.: Production of Local Skin Reactivity by Passive Transfer of Antiprotein Sera, Proc. Soc. Exper. Biol. & Med. **52**: 238, 1943.
5. Dale, H. H.: Some Chemical Factors in the Control of the Circulation, Lancet **216**: 1285, 1929.
6. Nicoll, P. A., and Campbell, D. H.: Anaphylaxis in the Surviving Intestine, J. Immunol. **39**: 89, 1940.
7. Dale, H. H.: A System of Bacteriology. His Majesty's Stationery Office, London **9**: 229, 1931.
8. Friedman, E.: A Cellophane Straw Used as an Ink-Writing Pointer in Kymographic Work, J. Lab. & Clin. Med. **26**: 1650, 1941.
9. Cole, A. G.: Preparation of Crystallized Egg Albumin (Ovalbumin), Proc. Soc. Exper. Biol. & Med. **30**: 1162, 1933.
10. Mirsky, I. A., and Wasserman, P.: In discussion of paper by Goldman, L.: The Effect of Fever Artificially Induced on the Skin Sensitivity of Guinea Pigs to Turpentine, J. Invest. Dermat. **2**: 281, 1939.

STUDIES ON RICKETTSIAL AGGLUTINATION IN TYPHUS

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THE first report of the development of rickettsial agglutinins as the result of infection was made by Otto and Dietrich,[1] who found that the sera of men and rabbits convalescent from typhus agglutinated a rickettsial suspension. Other early workers[2] confirmed this finding, using the same antigen, a suspension prepared from infected lice. In more recent years, with the development of methods for rickettsial multiplication apart from the insect vectors,[3-5] our knowledge concerning agglutinins has been amplified. It is now known that agglutinins develop in man and animals receiving rickettsial vaccines.[6-9] Information with respect to cross-reactions between the murine and epidemic strains, and whether one can distinguish between these infections, is less complete. Using a murine antigen prepared from rats[3] and an infected louse suspension for epidemic antigen, Zinsser and Castañeda[10] showed that sera from human convalescents from both types of typhus gave cross-agglutination. These authors noted that a higher cross-titer was obtained when murine convalescent serum was tested with epidemic antigen than when European serum was set up with murine antigen. Reports on investigations on the absorption of agglutinins from typhus antisera are limited to those of Castañeda and Kligler and their co-workers. It was observed by the former[11] that absorption of typhus serum with a suspension of *Bacillus proteus* removed only the proteus agglutinins, but that if the same serum were absorbed with rickettsial antigen, both proteus and rickettsial agglutinins were removed. Kligler and Oleinik[12] reported that absorption with either epidemic or murine antigen did not remove heterologous agglutinins completely.

The present report deals with the results of agglutination tests obtained over a period of years with a variety of sera from man and animals infected with or vaccinated against typhus, with the cross-agglutinations encountered, and with serum absorption experiments.

METHODS AND MATERIALS

Some of the agglutinations were performed with the tissue culture antigen previously described,[13] but for the most part the antigens used were prepared from the yolk sacs of infected eggs.[5] Seven-day-old embryos were inoculated with a 1:300 or 1:400 dilution of murine (Wilmington strain) or epidemic (Breinl strain) typhus yolk sac and incubated at 34 to 35° C. The majority of deaths occurred on the sixth and seventh days. Once it became apparent that the chicks were beginning to die, the eggs were candled every two or three hours and the dead ones were kept in the icebox until harvested. The harvested yolk sacs were then shaken by hand in bottles containing glass beads until a smooth suspension was obtained. Physiologic saline containing 0.2 per cent formalin was added to make a 20 per cent suspension and the material was placed at from 2 to 5° C. for from three days to a week, during which time

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Received for publication, April 6, 1945.

it was shaken every day. Microscopic examination revealed that freshly prepared suspensions usually contained cells packed with rickettsiae. In spite of the presence of formalin, autolysis of these cells took place when suspensions were allowed to remain in the icebox, with the consequent liberation of rickettsiae. The suspension was then filtered through gauze to remove any pieces of membrane and extracted in the cold with an equal volume of ether. About eighteen hours later the antigen was drawn off. As a preservative, phenyl mercuric nitrate sufficient to make a final concentration of 1:50,000 was added.

For the agglutination test, glass slides having twelve depressions were used. The serum to be tested was set up in tubes in doubling dilutions and one drop of each dilution was transferred to the slide with a capillary pipette. Using a capillary of the same bore, an equal volume of antigen was added. The slides were then rotated by hand to secure thorough mixing and placed on moist paper in Petri dishes in the incubator at a temperature of 40° C. Although lower temperatures may be employed, agglutination, especially of weakly positive sera, is delayed. For uniform results the slides were left in the incubator for five hours. They were then placed in the icebox overnight, after which a final reading was made with the low-power objective of an ordinary microscope (×100). Actually, with sera which show a titer of 1:80 or above, agglutination in the lower dilutions is visible to the unaided eye after incubation for from thirty to sixty minutes and in much less time if the rotation of the slide is carried out for two minutes. Where the test is being used for diagnostic purposes, this rapid agglutination of strongly positive sera is of practical importance. Positive and negative serum controls were included on each test. In the absence of agglutination, there is a perfectly smooth distribution of the antigen, which is easily distinguished from even a 1 plus agglutination reading. Any degree of agglutination may be considered significant, since, as will be pointed out, normal sera give no trace of agglutination whatever. Complete agglutination, where the entire field was covered by large clumps, was recorded as a 4 plus reading. In a 3 or 2 plus reaction the clumps were relatively smaller, and there was more space between them. In a reading recorded as a trace or 1 plus, the agglutinated particles were definite but of smaller size than in the case of a 2 plus reaction.

Proteus antigens were prepared fresh as needed from eighteen-hour cultures of *Bacillus proteus* OX19, Lister strain, at a density equal to the No. 3 tube of the McFarland nephelometer. For the agglutination test, 0.5 c.c. portions of antigen suspension were added to equal quantities of the serum dilutions. The tubes were incubated for two hours at 40° C. and placed in the icebox overnight before a final reading was made.

For absorption studies of the sera, 30 per cent antigen was used. Equal parts of antigen were combined with serum diluted 1:2.5 and the mixtures incubated for three hours at 37° C. Following fifteen minutes' centrifugation at 2,000 r.p.m., the supernatant was once more combined with an equal amount of antigen and incubation and centrifugation carried out as before. If necessary this procedure was repeated.

RESULTS

Human Studies.—

Typhus Cases and Convalescents: Hudson[14] found no positive reactions among twenty-five sera from normal adults in Ohio tested against murine typhus antigen. We have never seen typhus agglutinins in sera other than those

of persons with a history of typhus or of vaccination. The sera of seventy-five blood donors from a Philadelphia suburb were completely negative when tested for murine typhus agglutinins. (About 50 per cent had agglutinins for proteus OX19 in dilutions of 1:20 or 1:40.) The sera of thirty-five other normal adults from the Philadelphia area showed no agglutinins for epidemic antigen. By contrast, among 155 sera* from persons in Alabama with a clinical history of typhus within two years before bleeding, 104 gave positive results with murine antigen. It is possible that those giving negative results did not have typhus or that their agglutinins had disappeared. Among the positives, titers varied from 1:20 to 1:1280. In fifty-eight of the 104 positives, proteus agglutinins were absent or lower than those for typhus, showing a tendency in man, previously observed in rabbits,[15] toward a persistence of rickettsial agglutinins. In eighteen of the 104 patients the proteus agglutinins were higher than those for murine antigen. In some cases, no doubt, this might be a manifestation of an increase of proteus agglutinins normally present. It may be noted here that a pool of twenty of the sera showing the highest murine rickettsial titer gave no agglutination with spotted fever antigen.

Two cases of suspected epidemic typhus in persons exposed to infection during the production of typhus vaccine[16] were diagnosed beyond doubt by the increase in titer of the rickettsial agglutinins above the level induced by vaccination. This rise in titer occurred in one case on the fifth day of fever, and in the other on the eighth day of illness when the temperature had been normal for two days. Heterologous agglutinins (murine typhus) were also present, as was to be expected, but in lower titer than that of the infecting strain. The serum studies on these two cases are summarized in Tables I and II.

TABLE I. AGGLUTINATION TESTS ON SAMPLES OF SERUM FROM CASE 1 (EPIDEMIC TYPHUS)

SERUM SAMPLE	DILUTION OF SERUM													
	PROTEUS OX19					EPIDEMIC ANTIGEN				MURINE ANTIGEN			
	20	40	80	160	320	20	40	80	160	20	40	80	160
Before vaccination	3*	2	1	±	–	0	0	0	0	–	–	–	–
After 3 c.c. vaccine†	3	2	1	±	–	1	±	0	0	±	0	0	0
5th day of fever‡	3	3	2	1	0	2	2	1	0	1	±	0	0
6th day of fever	3	3	2	1	0	3	2	1	0	1	±	0	0
13th day after onset	4	4	4	3	3	3	3	3	1	3	3	2	2
31st day after onset	4	4	3	2	±	4	4	3	2	4	3	2	1
54th day after onset	4	4	2	2	±	4	4	3	1	3	2	1	±
109th day after onset	3	2	1	0	0	3	2	2	1	2	1	±	0

*± denotes trace of agglutination; 1, 2, 3, 4, degree of agglutination; 0, negative; – not done.

† Bleeding taken ten days after third dose.

‡Blood infectious for guinea pigs on this day (two months after vaccination).

TABLE II. AGGLUTINATION TESTS ON SAMPLES OF SERUM FROM CASE 2 (EPIDEMIC TYPHUS)

SERUM SAMPLE	DILUTION OF SERUM													
	PROTEUS OX19				EPIDEMIC ANTIGEN						MURINE ANTIGEN			
	20	40	80	160	20	40	80	160	320	640	20	40	80	160
Before vaccination	2	1	0	0	0	0	0	0	–	–	0	0	0	0
After 4.5 c.c. vaccine*	2	1	0	0	2	1	1	0	0	0	2	1	0	0
1st day of fever†	2	1	0	0	2	0	0	0	0	–	–	–	–	–
6th day after onset‡	2	1	0	0	2	0	0	0	–	–	–	–	–	–
8th day after onset	3	2	1	±	3	3	2	2	2	1	4	3	2	1
11th day after onset	3	2	1	±	4	3	3	3	3	2	4	3	3	2
70th day after onset	2	1	0	0	4	4	3	2	1	0	4	3	2	1

*Bleeding was taken five weeks after fifth dose.

†Ten weeks after vaccination.

‡Blood not infectious for guinea pigs on this day.

*These sera were obtained for us by Dr. Samuel R. Damon, of the State Board of Health Laboratories, Montgomery, Ala., to whom we express our thanks.

Vaccinated Subjects: During the course of epidemic typhus vaccine production in these laboratories, more than ninety persons were vaccinated. Some received three 1 c.c. doses of centrifuged yolk sac vaccine[16] which, on smear, showed very few rickettsiae. The others were given three or five 1 c.c. doses of ether-extracted vaccine made from rich yolk sac material. Samples of serum* were obtained from thirty-five of the subjects before vaccination, but this step was later omitted due to the pressure of other work. Serum was collected ten days after the last dose in most instances. The agglutinin responses for proteus OX19 and for epidemic typhus rickettsiae were studied. Space does not permit the tabulation of results, but the following facts were brought out in this study: (1) that regardless of the type of vaccine used, or the amount, there was considerable variation in individual response; (2) that the more potent vaccine in most cases gave higher agglutinin titers; (3) that negative rickettsial agglutination was found in only one instance; a poor response (1 plus agglutination at a 1:20 dilution of serum only) in six additional cases. These subjects were all in the group that had received the vaccine which, on smear, showed few rickettsiae. In those instances where blood was obtained after the third and again after the fifth dose, there was usually found to be no increase in titer at the second bleeding. In the cases where a comparison could be made with the prevaccination bleeding, the proteus agglutinins elicited by vaccination were not significant.

Animal Studies.—The formation of rickettsial agglutinins as the result of infection has been reported for rabbits,[1, 15] guinea pigs,[10] and monkeys.[7] Except in the case of rabbits, no observations have been reported on the time of appearance and the titers obtained in animals. It was felt, therefore, that pertinent information might be revealed by a study of the agglutinin response of animals following infection and vaccination and that the results might have practical application. Guinea pigs, mice, wild rats, and hamsters were studied.

Wild Rats: Philip and Parker[18] reported that in the case of laboratory rats the brain was infectious for 370 days. Unfortunately, the duration of agglutinins in this animal has not been determined and it is not known whether or not this antibody outlasts the period of infectivity of the rat brain.

Twenty-three sera from infected rats caught in Savannah† were set up with proteus OX19 and with murine and epidemic typhus antigens. Fifteen of the sera were positive for rickettsial agglutinins; only one had clear-cut proteus agglutinins. It was also found that where agglutinins for murine antigen were present there was usually agglutination of the heterologous suspension as well, although always in lower titer.

Guinea Pigs: In guinea pigs, considerable variation was encountered. In a group of animals receiving the same infectious material or vaccine the agglutinin response varied widely. The individual responses of guinea pigs inoculated with epidemic passage brain and vaccine, respectively, are recorded in Table III.

In guinea pigs infected with epidemic typhus, the peak of agglutinins was reached about ten days after defervescence. In animals bled on the fifth or sixth day of fever (eleventh or twelfth day after inoculation) no agglutinins were detectable.

In vaccinated pigs the appearance of agglutinins was also delayed. Titers were low and dropped rather rapidly. Better agglutination was found two weeks after vaccination than after three weeks.

*We are indebted to Dr. Leroy Wenger for his cooperation in performing these venipunctures.

†These were obtained by Dr. George Brigham, of the U. S. Public Health Service, and kindly forwarded to us by Dr. Ida Bengtson, of the National Institute of Health.

TABLE III. AGGLUTININS IN THE SERA OF GUINEA PIGS INJECTED WITH EPIDEMIC TYPHUS VACCINE OR BRAIN SUSPENSION*

GUINEA PIG	INOCULUM†‡	DILUTION OF SERUM			
		4	8	16	32
1	Vaccine	3	2	0	0
2	Vaccine	4	3	2	0
3	Vaccine	2	1	0	0
4	Vaccine	3	3	2	0
5	Vaccine	2	1	0	0
6	Vaccine	2	1	±	0
		DILUTION OF SERUM			
		20	40	80	160
1	Infected brain	2	±	0	0
2	Infected brain	4	3	3	3
3	Infected brain	4	3	3	2
4	Infected brain	4	4	3	3
5	Infected brain	3	3	2	1
6	Infected brain	4	3	2	1

*Blood taken two weeks after inoculation.
†Animals received two 0.5 c.c. doses of vaccine.
‡Animals received 1 c.c. 5 per cent brain suspension.

In guinea pigs infected with murine typhus (guinea pig tunica suspension) agglutinins developed much earlier than in pigs inoculated with brain suspension of the epidemic strain, but the peak was reached about the same time in both cases. It remained to be shown whether this was a strain characteristic or was due to the greater number of rickettsiae present in the endemic passage inoculum. Two guinea pigs each were infected with a 1:100 dilution of comparable yolk sacks of the two strains and bled on the fourth and sixth days of fever and on the fourteenth day after inoculation. As is seen in Table IV, there was no strain difference as to the formation of agglutinins under these conditions, and for both types of typhus titers were higher than usually found when guinea pig passage material was used as infectious inoculum.

TABLE IV. AGGLUTINATION TESTS ON THE SERA OF GUINEA PIGS INFECTED WITH 1 C.C. OF A 1:100 DILUTION OF MURINE OR EPIDEMIC YOLK SAC SUSPENSION*

GUINEA PIG	STATUS	ANTIGEN	DILUTION OF SERUM			
			20	40	80	160
M. T.† 1	4th day of fever	Murine	4	4	4	3
M. T. 2	4th day of fever	Murine	4	4	3	2
M. T. 1	6th day of fever	Murine	4	4	4	3
M. T. 2	6th day of fever	Murine	4	4	4	3
M. T. 1	14th day of disease	Murine	3	4	4	4
M. T. 2	14th day of disease	Murine	4	4	4	3
E. T.‡ 1	4th day of fever	Epidemic	4	4	3	2
E. T. 2	4th day of fever	Epidemic	4	4	4	3
E. T. 1	6th day of fever	Epidemic	4	4	3	2
E. T. 2	6th day of fever	Epidemic	4	4	4	3
E. T. 1	14th day of disease	Epidemic	4	4	4	4
E. T. 2	14th day of disease	Epidemic	4	4	4	4

*It may be noted here that small amounts of precipitins to yolk sac may be formed in guinea pigs, but the reactions are weak and irregular and do not obscure the results obtained in an experiment such as the above. A 1 plus at the 1:20 dilution of serum was the highest titer found in these guinea pigs with normal yolk sac antigen.
†M. T. denotes pigs infected with murine yolk sac.
‡E. T. denotes pigs infected with epidemic yolk sac.

Mice: Experiments with mice (Swiss, albino) showed these animals to be very satisfactory for agglutinin study. Sera from fourteen normal mice had no agglutinins for either typhus antigen. Agglutinins were found to be present as early as five days after intravenous inoculation of these animals with 0.5 c.c. of epidemic typhus yolk sac suspension at a 1:400 dilution. By using murine typhus yolk sac as inoculum and injecting by the intraperitoneal route with 0.5 c.c. of a 1:400 dilution, it was possible to detect agglutinins on the fifth,

but not on the fourth, day. By the seventh day much higher titers were found with both strains (Table V). Vaccination of mice with vaccines of both types of typhus[19] produced in each case good agglutinin response by the seventh day, although, as might be expected, titers were not as high as when living rickettsiae were injected.

TABLE V. AGGLUTININS IN THE SERA OF MICE RECEIVING TYPHUS YOLK SAC*

MOUSE	INFECTING STRAIN	ROUTE†	DILUTION OF SERUM			
			20	40	80	160
1	Epidemic	i.v.	3	2	1	0
2	Epidemic	i.v.	4	3	2	1
3	Epidemic	i.v.	4	3	3	2
4	Murine	i.p.	4	3	2	2
5	Murine	i.p.	4	3	2	0
6	Murine	i.p.	3	2	1	0

*Blood obtained one week after the inoculation of 1:400 yolk sac suspension.
†I.V. Intravenously; I.P. intraperitonealy.

Hamsters: Hamsters were found to be very resistant to inoculation with epidemic typhus.[20] The intraperitoneal injection of 1 c.c. of a 10 per cent yolk sac suspension produced no febrile reaction and very feeble rickettsial agglutinins when tests were made eight days after inoculation. Because of their lack of susceptibility to infection, further work with these animals was not undertaken.

Cross-Agglutination.—In all of the species studied, sera from animals inoculated with infectious material gave agglutination with the heterologous typhus antigen with but one exception. This occurred in the case of mice infected intravenously with epidemic typhus (*vide infra*).

TABLE VI. HOMOLOGOUS AND HETEROLOGOUS AGGLUTINATION TESTS ON HUMAN SERA FROM CASES OF MURINE TYPHUS*

CASE	ANTIGEN	DILUTION OF SERUM							
		20	40	80	160	320	640	1280	2560
1	Murine	4	4	4	4	3	2	1	±
1	Epidemic	3	3	2	2	1	0	0	0
2	Murine	4	4	4	4	3	2	0	0
2	Epidemic	4	3	2	1	0	0	0	0
3	Murine	4	4	4	4	3	3	2	1
3	Epidemic	4	4	3	2	2	1	±	0

*The sera of these patients and twenty other convalescent murine typhus patients tested showed no agglutination with spotted fever antigen.

Examples of cross-agglutination in human cases of epidemic typhus were given in Tables I and II. In human sera from the endemic disease, the heterologous agglutinins were likewise present but again not in sufficiently high titer to confuse the diagnosis. The reactions observed with sera from three patients with murine typhus, using both types of antigen, are shown in Table VI.

In the case of guinea pigs, cross-agglutination was evident once the homologous antibody was present in more than minimal titer. This is illustrated in Table VII, which shows the cross-agglutination found for passage pigs that had received either epidemic brain suspension or murine tunica.

TABLE VII. CROSS-AGGLUTINATION IN THE SERA OF GUINEA PIGS

GUINEA PIG	INFECTING STRAIN	ANTIGEN	DILUTION OF SERUM					
			20	40	80	160	320	640
1	Epidemic	Epidemic	4	4	4	4	3	2
1	Epidemic	Murine	3	2	2	1	0	0
2	Murine	Murine	4	4	4	3	2	2
2	Murine	Epidemic	3	2	0	0	0	0

Cross-agglutination was found in the sera of wild rats as mentioned previously. It did not occur where the homologous antibody had dropped to a low titer.

TABLE VIII. CROSS-AGGLUTINATION IN THE SERA OF MICE RECEIVING MURINE OR EPIDEMIC INFECTIOUS YOLK SAC*

MOUSE	INFECTING STRAIN	ROUTE†	DILUTION OF SERUM							
			MURINE ANTIGEN				EPIDEMIC ANTIGEN			
			20	40	80	160	20	40	80	160
1	Murine	I.P.	4	3	2	2	4	3	2	1
2	Murine	I.P.	4	3	2	0	3	2	1	0
3	Murine	I.P.	3	2	1	0	2	0	0	0
4	Murine	I.P.	2	1	±	0	2	±	0	0
5	Murine	I.P.	3	2	1	0	3	1	±	0
6	Murine	I.P.	4	3	2	1	3	2	1	0
1	Epidemic	I.V.	0	0	0	0	3	3	2	1
2	Epidemic	I.V.	0	0	0	0	3	2	2	1
3	Epidemic	I.V.	0	0	0	0	3	2	1	0
4	Epidemic	I.V.	±	0	0	0	4	3	3	2
5	Epidemic	I.V.	±	0	0	0	4	3	3	2
6	Epidemic	I.V.	±	0	0	0	4	3	2	1

*Blood obtained one week after inoculation of 1:400 yolk sac suspension.
†I.P. Intraperitonealy; I.V. intravenously.

In mice inoculated intraperitoneally with virulent murine yolk sac material diluted from 1:400 to 1:1000, heterologous agglutinins were present by the seventh day, at which time the homologous titer was usually high (Table VIII). In mice infected by the intravenous route with epidemic typhus yolk sac at a 1:400 dilution, a good titer of homologous agglutinins was likewise in evidence by the seventh day, but no cross-agglutination occurred (Table VIII). The result was the same for all mice infected with epidemic typhus intravenously; cross-agglutination was almost entirely negative. This conceivably might be due to strain difference or might be a function of the route of administration. To determine which of these hypotheses was correct, groups of mice were injected with epidemic infectious material, using the intraperitoneal route. Some cross-agglutinins resulted from this treatment but they did not equal the titers found in mice given murine typhus material by the same route (Table VIII), so that the phenomenon must at least in part be due to a strain difference as regards the mouse. Smears made at autopsy on numerous mice as early as two and one-half hours after intravenous inoculation with epidemic yolk sac material disclosed that the rickettsiae had disappeared. On rare occasions a very few organisms were found in smears made from spleen and blood.

Agglutinin Absorption Tests.—Agglutinins were readily absorbed from immune serum. With a 30 per cent rickettsial suspension, it was found that two or three absorptions (depending on the original titer of the serum) usually would remove all of the agglutinins completely for the antigen used, leaving the heterologous antibody intact. The results obtained when murine convales-

TABLE IX. AGGLUTININ ABSORPTION OF MURINE GUINEA PIG SERUM WITH EPIDEMIC ANTIGEN

	DILUTION OF SERUM			
	20	40	80	160
Murine serum + Murine antigen before absorption	4	4	3	2
Murine serum + Epidemic antigen before absorption	4	3	2	1
Murine serum + Murine antigen after 1st absorption	4	4	3	2
Murine serum + Epidemic antigen after 1st absorption	3	2	1	0
Murine serum + Murine antigen after 2nd absorption	4	4	3	2
Murine serum + Epidemic antigen after 2nd absorption	±	0	0	0
Murine serum + Murine antigen after 3rd absorption	-	4	3	2
Murine serum + Epidemic antigen after 3rd absorption	-	0	0	0

cent passage guinea pig serum was absorbed with the heterologous antigen are shown in Table IX. With sera from human patients with endemic typhus, it was found that absorption with proteus antigen left the rickettsial agglutinins intact even after six absorptions. Absorption of the same sera with murine rickettsial antigen removed both proteus and rickettsial antibodies.

DISCUSSION

The results of the serologic tests in both man and animals indicate that the rickettsial slide agglutination test using a concentrated antigen may be a useful diagnostic aid in studies on typhus. To be of use, however, a diagnostic agent must be shown to be specific and to give negative results with known normal sera.

With the test as performed in the foregoing experiments, no agglutination with either typhus antigen was encountered in numerous samples of normal serum from man and animals. In addition, sera from twenty cases of murine typhus tested singly and twenty others with high murine titers, tested as a pool, gave no agglutination with spotted fever antigen. Furthermore, we have found no agglutinins for typhus antigen in fifteen pools of sera from rabbits hyperimmunized with large quantities of live spotted fever rickettsiae, each pool containing the sera of from fourteen to eighty-two rabbits. The antigens employed may therefore be considered to have fulfilled accepted criteria for specificity.

The specificity of the rickettsial agglutination test has been seriously questioned by Plotz and co-workers[21] in a recent report, in which sera from thirteen of fourteen cases diagnosed as spotted fever were found to give agglutination with typhus antigens, using a macroscopic tube agglutination technique. The details of the technique used were not described and the results with spotted fever antigen were not given. This report is at variance not only with the work described in this paper where the test was found to be specific, but also with unpublished observations in this laboratory on the sera of spotted fever cases, and is therefore puzzling.

The usual procedure for the detection of typhus in a rat population (i.e., the inoculation of rat brains into guinea pigs) is tedious and expensive. Recently, Brigham and Bengtson[22] have suggested the use of the complement fixation test to detect murine typhus in rats as an aid in rat control measures. The results of our studies on the human and rat sera from two of our southern states suggest that city or county surveys for the presence of typhus could be made equally as well by the simple means of the agglutination test.

In mild cases of human typhus, the agglutination test has been found particularly useful. Here the clinical diagnosis is difficult and the isolation of typhus rickettsiae from the patient's blood is a lengthy procedure which may give negative results. Even when positive results are obtained, guinea pigs must be reinfected with known typhus material to prove their original infection to have been due to typhus, and the long-delayed diagnosis becomes one of academic interest only. The agglutination test, requiring but one reagent, offers a simple and rapid method for early diagnosis and for the differentiation of strains of this infection from each other and from spotted fever. In investigative work also, the agglutination test has been found useful for the diagnosis of mild or inapparent typhus infections in the guinea pig, particularly in the epidemic variety of the disease. In this infection, unlike murine typhus and spotted fever in guinea pigs, no scrotal involvement occurs to guide the investigator in the interpretation of a febrile state and many experiments must be repeated because of concurrent infection.

The amount of cross-agglutination occurring in sera has been found to depend on the height of the homologous titer in the serum being studied. When serum dilutions were carried out to the end point, there was usually no difficulty in deciding which strain of typhus was the cause of the infection. Generally speaking, the murine sera gave better cross-agglutination than did the epidemic sera.

The results of absorption tests confirm Castañeda and Zia's[11] observation. The absorption experiments were clear cut, and removal of heterologous agglutinins was complete providing that absorption was carried out a sufficient number of times.

The test, though time consuming, may be used with relative ease due to the availability of rich yields of rickettsiae in yolk sacs and may be employed to advantage in the rare cases where the titers of the patient's serum are almost the same for both antigens.

SUMMARY

The microscopic agglutination test as used was found to be an easy and convenient means for the diagnosis of typhus in human sera and proved to be a useful tool for the study of agglutinin development in man and animals following infection or vaccination. The method has been found to be simple and specific. Cross-agglutination reactions are discussed. The observations of Castañeda on the removal of heterologous typhus agglutinins were confirmed. Sera from rabbits infected with spotted fever did not agglutinate either of the typhus antigens. Sera obtained from cases of endemic typhus were found not to agglutinate spotted fever antigen. The procedures for the preparation of the antigens and for carrying out the tests are described.

Since this manuscript was completed, results of agglutination tests using a mouse lung antigen have been published (Castañeda, M.: J. Immunol. **50:** 179, 1945) which corroborate the findings in this paper.

REFERENCES

1. Otto, R., and Dietrich: Beitrage zur Rickettsienfrage, Deutche. med. Wchnschr. **43:** 577, 1917.
2. Kolle and Wassermann: Handb. d. Path. Mikr. pp. 1190-92, 1930.
3. Zinsser, H., and Castañeda, M. R.: A Method of Obtaining Large Amounts of Rickettsiae by X-ray Radiation of Rats, Proc. Soc. Exper. Biol. & Med. **29:** 840, 1932.
4. Zinsser, H., Wei, H., and Fitzpatrick, F. K.: Agar Slant Tissue Cultures of Typhus Rickettsiae, Proc. Soc. Exper. Biol. & Med. **37:** 604, 1937.
5. Cox, H.: Use of Yolk Sac of Developing Chick Embryos as Medium for Growing Rickettsiae of Rocky Mountain Spotted Fever and Typhus Groups, Pub. Health Rep. **53:** 2241, 1938.
6. Liu, P., Zia, S., and Wang, K.: Serological Studies on Subjects Vaccinated Against Typhus, Proc. Soc. Exper. Biol. & Med. **38:** 682, 1938.
7. Fitzpatrick, F.: Vaccination of Monkeys With Agar Tissue Cultures of European Rickettsiae, Proc. Soc. Exper. Biol. & Med. **42:** 217, 1939.
8. Fitzpatrick, F.: Vaccination Against Spotted Fever With Agar Tissue Cultures, Proc. Soc. Exper. Biol. & Med. **42:** 219, 1939.
9. Fitzpatrick, F.: Antibody Production in Man Following Spotted Fever Vaccine, Abs. J. Bact. **43:** 93, 1942.
10. Zinsser, H., and Castañeda, M.: On the Serum Reactions of Mexican and European Typhus Rickettsiae, J. Exper. Med. **56:** 455, 1932.
11. Castañeda, M. R., and Zia, S.: Antigenic Relation Between Proteus X19 and Typhus Rickettsiae, J. Exper. Med. **58:** 55, 1933.
12. Kligler, I., and Oleinik, E.: Specific Diagnosis of Typhus Fever by Rickettsial Agglutination, Nature **152:** 627, 1943.
13. Zinsser, H., Fitzpatrick, F., and Wei, H.: A Study of Rickettsiae Grown on Agar Tissue Cultures, J. Exper. Med. **69:** 179, 1939.
14. Hudson, N.: Macroscopic Agglutination Test With Typhus Rickettsiae Prepared From Infected Rodent Lungs, J. Infect. Dis. **67:** 227, 1940.
15. Fitzpatrick, F., and Hampil, B.: Immunological Reactions in Rickettsial Diseases With Special Reference to the Time of Appearance of Antibodies, Am. J. Pub. Health **31:** 1301, 1941.

16. Gold, H., and Fitzpatrick, F.: Typhus Fever in a Previously Vaccinated Laboratory Worker, J. A. M. A. **119**: 1415, 1942.
17. Cox, H., and Bell, J.: Epidemic and Endemic Typhus: Protective Value for Guinea Pigs of Vaccines Prepared From Infected Tissues of the Developing Chick Embryo, Pub. Health Rep. **55**: 110, 1940.
18. Philip, C., and Parker, R.: Persistence of Viruses of Endemic Typhus, Rocky Mountain Spotted Fever and Boutonneuse Fever in Tissues of Experimental Animals, Pub. Health Rep. **53**: 1246, 1928.
19. Fitzpatrick, F.: Vaccination of Mice Against Typhus, Proc. Soc. Exper. Biol. & Med. **58**: 188, 1945.
20. Unpublished experiments.
21. Plotz, H., Bennett, B., Wertman, K., and Snyder, M.: Cross-Reacting Antibodies in Rocky Mountain Spotted Fever, Proc. Soc. Exper. Biol. & Med. **57**: 336, 1944.
22. Brigham, G., and Bengtson, I.: A Study of the Complement Fixation and Weil-Felix Reactions in Wild Rats as Related to the Isolation of the Virus of Endemic Typhus, Pub. Health Rep. **60**: 29, 1945.

THE BEHAVIOR OF BLOOD CHOLESTEROL IN THYROTOXIC PATIENTS UNDER TREATMENT WITH THIOURACIL

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THE influence of thiourea derivatives upon the blood cholesterol of thyrotoxic patients has been previously mentioned by two groups of observers.[1, 2] Jennings and his associates noted a rapid rise of the blood cholesterol in twelve patients who were taking thiourea. Stable levels were rapidly attained, from which only minor subsequent variations were observed. In their hands, thiouracil administered to a single patient for four months exerted a similar action upon the blood cholesterol.

We have estimated blood cholesterol in the fasting specimens of fifty-two of seventy-eight thyrotoxic patients and have made serial analyses in thirty-six of these. None of the thirty-six had a co-associated diabetes mellitus. Total cholesterol was determined by a method previously described by one of us (I. J. D.[3]). The normal range of values by this method is from 150 to 200 mg. per 100 c.c. of plasma. The free fraction of cholesterol was estimated by the Shoenheimer-Sperry technique[4] modified so as to require but 0.5 c.c. of serum for each analysis. All of these patients had clinical manifestations clearly confirming the toxic status of the thyroid.

The results of our study are summarized in Table I and Fig. 1.* No actual data for the free fraction of the blood cholesterol are included as this always varied directly with the total and represented from 25 to 35 per cent of that figure in any individual instance. In eleven of the fifty-two individuals the initial level for total cholesterol in the blood was above 200 mg. per 100 c.c. The averaged mean pretreatment basal metabolic rate for these eleven was +48.5 per cent with a low figure of +37 and a high of +64 per cent, respectively. These facts show that thyrotoxicosis may and does occur in the presence of

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Received for publication, March 15, 1945.

*All the supplies of thiouracil used in these studies were supplied by Drs. Benjamin W. Carey and Stanton M. Hardy, of the Lederle Laboratories, Pearl River, N. Y. Their courtesy is herewith gratefully acknowledged.

hypercholesterolemia. Therefore, the fasting blood level of this substance cannot be relied upon as an infallible index for either the diagnosis of thyrotoxicosis or its treatment.

TABLE I. RELATION OF ABSOLUTE VALUES FOR CHOLESTEROL (MG. PER 100 C.C. OF BLOOD) TO THE BASAL METABOLIC RATE IN FIFTY-TWO PATIENTS WITH THYROTOXICOSIS UNDER TREATMENT WITH THIOURACIL

	NUMBER OF CASES	BASAL METABOLIC RATE		
		15	BETWEEN 0 AND 10	BETWEEN 10 AND 15
Pretreatment	52			
Values above 200	11	11	--	--
After treatment	36			
Values above 200	26		14	12

However, in any single thyrotoxic individual, it has been repeatedly demonstrated that the blood level for cholesterol rises from its initial point as the basal metabolic rate falls (Fig. 1). If absolute values are used, it is impossible to plot an informative curve because of the wide variation from patient to patient in the actual concentrations of cholesterol in the blood. For instance, the lowest pretreatment value for total cholesterol was 91 mg. per 100 c.c. in a patient whose corresponding basal metabolic rate was +65 per cent; the highest pretreatment blood level for the same substance was 374 mg. per 100 c.c. in a nondiabetic patient with a corresponding basal metabolic rate of +42 per

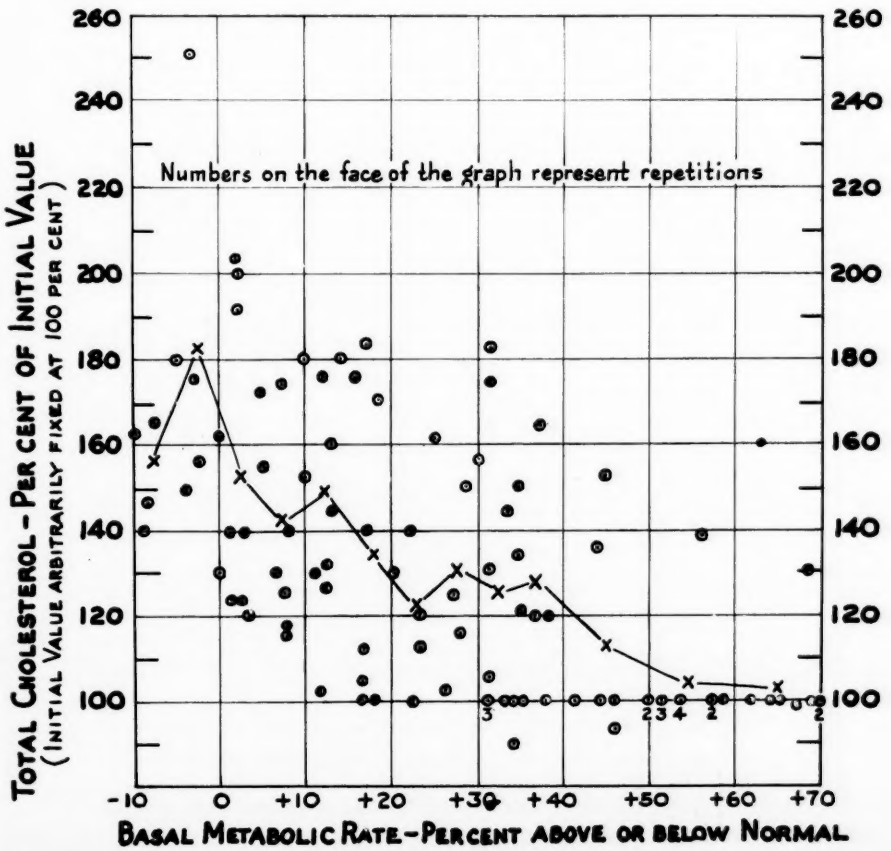

Fig. 1.—Percentual changes in cholesterol in relation to alterations in the basal metabolic rates of patients with thyrotoxicosis under thiouracil treatment (serial examinations in thirty-one patients).

cent. Of the fifty-two patients whose blood levels for cholesterol were studied prior to receiving thiouracil, four showed less than 100 mg. per 100 c.c.; eleven more than 200 mg. per 100 c.c. The initial level for total cholesterol in the blood lay between 100 and 149 mg. per 100 c.c. in sixteen patients and between 150 and 199 mg. per 100 c.c. in twenty-one patients.

Of the thirty-six fully controlled patients whose total blood cholesterol was estimated, four had post-treatment values between 150 and 199 mg. per 100 c.c. In twelve, the figures lay between 200 and 249 mg. per 100 c.c.; in thirteen, between 250 and 299 mg. per 100 c.c.; in five, between 300 and 389 mg. per 100 c.c.; and in two, were over 400 mg. per 100 c.c.

It is obvious from these data that no satisfactory curve could possibly be plotted from the absolute figures for the total cholesterol in the blood. However, when we arbitrarily fix the initial or pretreatment figure at 100 per cent and plot all others percentually therefrom, the "scattering" is not so great and the trend of the mean value for the blood cholesterol in all cases moves inversely with the basal metabolic rate (Fig. 1). A sufficient number of determinations have not been made to establish a mathematical curve with certainty, but a straight line relationship seems likely at least within values for the basal metabolic rate of from –10 to +70.

The averaged initial value for all determinations in fifty-two individuals was 170 mg. per 100 c.c. of blood; the averaged maximum value in thirty-six subjects, during or after treatment, was 245 mg. per 100 c.c. The mean basal metabolic rates corresponding to these two determinations were +48.8 and +11.6, respectively. From Table I it will be further noted that the blood cholesterol was high in 33.3 per cent of the patients when their basal metabolic rates lay between +10 and +15, while a second third (actually 38.8 per cent) developed values above 200 mg. per 100 c.c. when the metabolic rates fell between 0 and +10.

Such high values for cholesterol in the blood in the presence of normal or slightly elevated basal metabolic rates may indicate some aberration of fat metabolism or transport in the individual whose thyrotoxicosis has been relieved by thiouracil. It is our experience that these patients are clinically at their best when their basal metabolic rates range from +5 to +15.[2, 5] This conforms to the zone at which high cholesterol values begin to make themselves evident, an academically interesting fact with important obvious clinical implications.

Other practical applications of the blood cholesterol determination in the thiouracil-treated thyrotoxic patient are not wanting. In patients who suffer from claustrophobia or who for some other reason fear the taking of a basal metabolic rate, careful attention to the values for blood cholesterol may enable the clinician to obtain optimum results from therapy with thiouracil. For instance, successive basal metabolic rates at weekly intervals in a highly nervous, easily excitable patient (No. 31)[5] were +87, +76, +81 and +74. During this time there was a continual steady improvement in the clinical picture, and the values for blood cholesterol corresponding to the above metabolic rates were 112, 121, 183, and 240, respectively. Operation for nodular goiter was performed without difficulty and the postoperative hospitalization was short and uneventful.

SUMMARY AND CONCLUSIONS

1. Fasting blood cholesterol values above 200 mg. per 100 c.c. have been obtained in eleven of fifty-two patients with untreated thyrotoxicosis.

2. In thirty-six of these, serial examinations during treatment revealed a more or less straight-line relationship between the percentual elevation of the blood cholesterol and the falling basal metabolic rate.

3. Under treatment, high values for blood cholesterol were obtained in the majority of patients whose basal metabolic rates had been reduced to +10 or below, and in a considerable number in whom the rate lay between +10 and +15. Clinically, patients felt "at their best" when the basal metabolic rates lay between +5 and +15 and had concomitantly high normal values for blood cholesterol.

4. It is suggested that either the basal metabolic rate or the cholesterol value or a combination of the two may be used as a guide for the satisfactory management of thyrotoxicosis with the drug thiouracil.

REFERENCES

1. Jennings, L. M., Mawson, C. A., and Tindall, W. J.: Thiourea Therapy and Blood Cholesterol, Lancet **2**: 91-92, 1944.
2. McGavack, T. H., Gerl, A. J., Vogel, M., and Schwimmer, D.: The Treatment of 26 Thyrotoxic Patients With Thiouracil and a Review of Toxic Reactions in All (135) Reported Cases, J. Clin. Endocrinol. **4**: 249-261, 1944.
3. Drekter, I. J.: A New Method for the Direct Determination of Cholesterol, Bull. New York, M. Coll., Flower and Fifth Ave. Hosps. **6**: 138-141, 1943.
4. Shoenheimer, R., and Sperry, W. M.: A Micromethod for the Determination of Free and Combined Cholesterol, J. Biol. Chem. **106**: 745-760, 1934.
5. McGavack, T. H., Gerl, A. J., Morton, J. H., Vogel, M., and Schwimmer, D.: Observations on 78 Thyrotoxic Patients Treated With Thiouracil, J. Clin. Endocrinol. In press.

A NOTE ON THE IN VITRO RESPIRATION OF MUSCLE IN MYASTHENIA GRAVIS

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EXPERIMENTS previously reported[1] have suggested that the in vitro respiration of skeletal muscle from patients with myasthenia gravis may show certain abnormalities. The present paper describes a continuation of that type of investigation and is a report of experiments on the respiration of skeletal muscle tissue from two additional patients with this disease.

EXPERIMENTAL

The experiments consist of measurements of oxygen uptake of respiring muscle obtained at biopsy, and the effect of various supplements—dicarboxylic acids, cytochrome C, prostigmine, ephedrine, cocarboxylase, insulin, and "muscle juice"—on the respiration of the minced muscle. The details of technique have been described.[2] Dicarboxylic acid supplements were in final concentration of 0.003 M. The cytochrome was a purified sample of cytochrome C prepared according to the method of Keilin and Hartree[3]; 100 micrograms were used in each of the flasks that contained cytochrome. The concentrations of other supple-

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These studies were made in 1940 and publication deferred in the hope that additional observations would be made. Circumstances have prevented any further experimentation and it is thought desirable to publish them at this time, incomplete as they are.

Received for publication, April 27, 1945.

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ments per vessel were as follows: prostigmine bromide, 0.5 mg.; ephedrine sulfate, 0.5 mg.; cocarboxylase, 100 micrograms; insulin, 4 units; and muscle juice, 0.2 c.c. The muscle juice was prepared as previously described.[4] The total volume of the contents of the vessels was 2.8 c.c. All results are reported in cubic millimeters of O_2 per milligram of dry weight of tissue.

CASE HISTORIES AND RESULTS

CASE 1.—H. E., a woman physician, aged 48 years, had first experienced symptoms of myasthenia gravis twenty-one years previously. Intermittent diplopia was noticed five years later, and this was followed in the next year by weakness of the arms and legs and of other muscles innervated by the cranial nerves. The symptoms became worse following each of several upper respiratory infections. Her case was intensively studied at the Presbyterian, Michael Reese, and Billings Hospitals in Chicago, at the University of Minnesota Hospital, and at the Mayo Clinic. The diagnosis of myasthenia gravis was confirmed in each institution and various therapies were tried without success. For nearly six years she was bedridden and unable even to turn over. In 1930 she published her accidental discovery that ephedrine produced remarkable alleviation of her symptoms.[5] Using this drug she improved to such an extent that she was able to travel around the world. In recent years she has been almost normally active while taking 15 mg. of prostigmine bromide from four to six times daily, supplemented by ephedrine sulfate in doses of from 25 to 50 mg. daily.

The patient entered Billings Hospital to submit to a muscle biopsy. Her last dose of prostigmine (15 mg.) had been taken the evening of entry and she received no more until after the biopsy twenty-five hours later. The morning after admission she appeared in good health and normally active, her only difficulty being, as usual, in combing her hair. Her height was 168 cm.; weight, 74.3 kilograms; temperature, 98.6° F.; and pulse, 87. The muscles of the limbs were flabby and became fatigued on repeated movements. A slight bilateral ptosis of the eyelids was present but there was no wrinkling of the forehead. The eyes moved normally. Swallowing and speaking were well performed. There were no significant findings in the lungs, heart, or abdomen. The blood pressure was 118/78. All tendon reflexes were slightly hypoactive. The blood showed no abnormality except a mild hypochromic anemia. The urine was normal.

The day after admission the patient received no medication. In the afternoon she felt weak and very tired and ptosis of the lids was observed. She had some difficulty in helping herself onto the cart which took her to the operating room in the late afternoon where about 8 Gm. of fat-free muscle were removed from the gastrocnemius under local anesthesia. Great care was taken to avoid infiltration of the muscle itself, excision of which was therefore momentarily painful. The tissue was immediately placed in an ice-cold container and taken to the laboratory. On return to her room the patient received 30 mg. of prostigmine orally. Convalescence was uneventful.

TABLE I. THE EFFECT OF VARIOUS SUPPLEMENTS ON THE RESPIRATION OF MUSCLE IN MYASTHENIA GRAVIS—CASE 1 (H. E.)

SUPPLEMENTS	OXYGEN UPTAKE (TOTAL U_{O2} VALUES)			
	1ST HR.	2ND HR.	3RD HR.	4TH HR.
0.01 M glucose in buffer				
O—Control (Values are average of duplicate runs)	1.4	1.9	2.2	2.3
Succinate	2.5	5.2	7.8	9.0
Succinate + cytochrome	4.2	7.5	10.6	13.0
Cytochrome	2.4	4.9	7.3	8.7
Prostigmine	2.6	5.0	7.3	8.4
Succinate + prostigmine	3.7	6.8	9.4	11.7
Fumarate	2.6	5.4	8.2	10.5
Citrate	2.4	4.5	6.5	8.2
Glutamate	2.8	5.3	7.8	10.2
Muscle juice	3.0	5.8	8.6	10.2
Succinate + muscle juice	3.8	6.2	8.3	10.2
Succinate + cytochrome + prostigmine + cocarboxylase + insulin + muscle juice	4.5	8.2	11.9	15.0
As directly above but with no succinate	2.9	5.4	7.8	9.5
Succinate + cytochrome + prostigmine	4.0	7.3	10.4	13.0
Cytochrome + prostigmine	2.5	4.8	7.1	8.8

Results of the experiments on the respiration of this tissue are given in Table I and Fig. 1. The latter also shows for comparison results of a similar experiment with "normal human muscle" represented by a biopsy of the abdominal rectus removed during a cholecystectomy using spinal anesthesia. It is observed that the oxygen uptake of the unsupplemented "myasthenic muscle" was very low. The Q_{O_2} was 1.4, whereas we have found "normal human skeletal muscle" to have a Q_{O_2} averaging 3.2, a decrease from the "average normal" of 56 per cent. Furthermore, normal human muscle generally shows a linear rate of oxygen uptake for from one and one-half to two hours, while in this case the respiration fell off markedly after one hour. The addition of succinate, fumarate, citrate, glutamate, cytochrome C, prostigmine, or a muscle juice preparation resulted in an increase of oxygen uptake of about 86 per cent over the Q_{O_2} of the unsupplemented myasthenic muscle, and of 290 per cent over the four-hour U_{O_2}. Adding succinate and cytochrome, or succinate and prostigmine, gave rates of oxygen uptake similar to what might be expected with normal, unsupplemented human skeletal muscle. The increases here were of the order of 280 per cent over the Q_{O_2} of the unsupplemented myasthenic muscle, and 540 per cent over the four-hour U_{O_2}. The combination of cytochrome and prostigmine was no better than either substance alone.

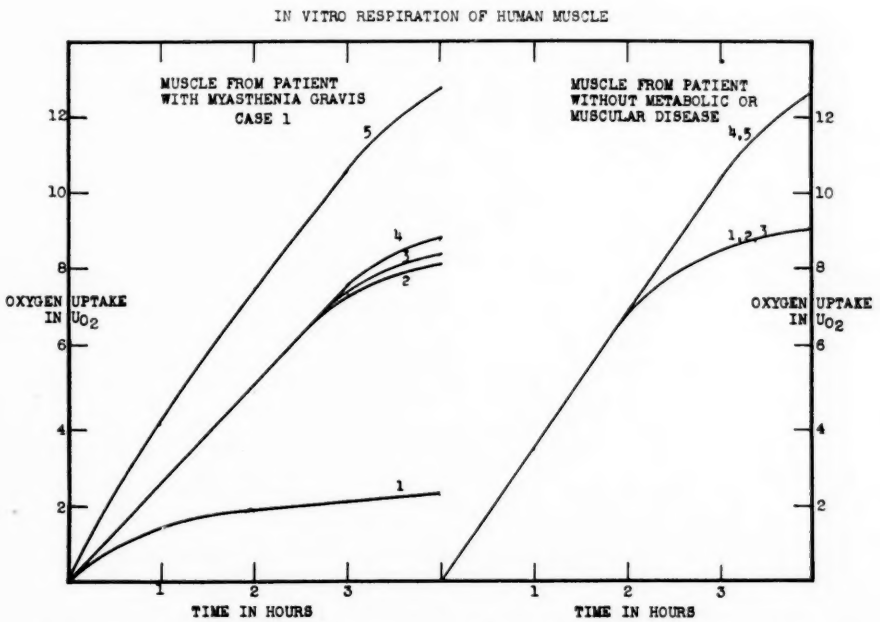

Fig. 1.—The effect of various supplements on the respiration of human muscle. *1*, Unsupplemented control; *2*, addition of prostigmine; *3*, addition of cytochrome C; *4*, addition of succinate; *5*, addition of succinate and cytochrome.

CASE 2.—A. N., a woman stenographer, aged 26 years, was admitted to Billings Hospital complaining of weakness and stiffness of the fingers and blurring of vision for ten months, fatigue on speaking and chewing for six months, generalized fatigue and inability to open or close her eyes completely for four months, and difficulty in swallowing with regurgitation of fluids through the nose for five weeks. Three weeks before admission she had been told by another physician that she had myasthenia gravis and had been given 6 tablets (presumably 15 mg. each) of prostigmine daily with considerable benefit. She had continued this medication until the day of her entry. Aside from moderate alcoholism the history contained no other relevant material.

General examination, performed after the patient had taken 60 mg. of prostigmine in the preceding eight to ten hours, showed a well-developed and well-nourished woman in no apparent distress. Her height was 163 cm.; weight, 51.7 kilograms; temperature, 100° F. (later normal); and pulse, 92. There were no abnormal findings in the heart, lungs, or abdomen. The blood pressure was 110/70. Five ecchymotic areas were observed over the tibias. Neurologic examination revealed weakness of the orbicularis oculi on both sides, and slight facial weakness. The speech was somewhat mumbling. Ocular convergence

and other extraocular movements were normal. The tone of the skeletal muscles was good. The tendon reflexes were normal. Routine laboratory studies gave essentially normal results except for a slight hypochromic anemia. X-ray of the chest showed no evidence of enlargement of the thymus. The basal metabolic rate, taken forty-one hours after prostigmine and sixteen hours after ephedrine, was –7 per cent.

After admission, prostigmine was withheld for twenty-two hours. At the end of this time there was marked ptosis of the eyelids, which could not be completely closed. Retraction of the lips was limited. The voice became nasal when the patient counted to 70. Definite weakness of the skeletal muscles, especially the deltoids, was observed. Tests with the dynamometer showed R 30, L 27. The patient was then given 0.5 mg. of prostigmine intramuscularly. Within twenty minutes, definite, and in some instances marked, improvement in the above functions took place, and at thirty minutes the dynamometer readings were R 62, L 56.

For the next three days the patient received from 50 to 75 mg. of ephedrine sulfate daily but no prostigmine. The voice became nasal, hand grasp very weak, smile snarling, and fatigue marked. On the fourth day she received neither drug until after the biopsy of the gastrocnemius which was performed late that morning. The surgical procedure and the treatment of the tissue were the same as in Case 1 except for the preoperative administration of 0.18 Gm. of calcium nembutal per rectum. The results are given in Table II. These data differ somewhat from those in Case 1. The oxygen uptake of the unsupplemented myasthenic muscle was higher than in the preceding experiment, but it was still lower by about 18 per cent than values obtained with normal human muscle. The only single supplement which increased the oxygen uptake appreciably was a dicarboxylic acid, fumarate. Neither prostigmine nor ephedrine alone gave a significant change; however, their addition together resulted in a prolongation of the initial rate of oxygen consumption so that the four-hour U_{O_2} was increased by 22 per cent. Cytochrome C alone was without effect, and its addition with fumarate did not stimulate respiration any more than did the latter substance by itself. The data obtained using a pyruvate substrate were similar to those with glucose, except that here prostigmine alone gave an increase in the four-hour oxygen uptake of 24 per cent.

TABLE II. THE EFFECT OF VARIOUS SUPPLEMENTS ON THE RESPIRATION OF MUSCLE IN MYASTHENIA GRAVIS—CASE 2 (A. N.)

SUPPLEMENTS	OXYGEN UPTAKE (TOTAL U_{O_2} VALUES)			
	1ST HR.	2ND HR.	3RD HR.	4TH HR.
0.01 M glucose in buffer				
O—Control (Values are average of duplicate runs)	2.8	5.3	7.3	8.6
Fumarate	3.6	8.5	12.6	15.5
Fumarate + cytochrome	3.7	8.6	12.3	15.5
Cytochrome	2.9	6.2	8.4	9.4
Prostigmine	2.7	5.6	8.2	9.3
Fumarate + prostigmine	3.8	8.8	12.1	15.1
Ephedrine	2.5	5.1	7.1	8.2
Ephedrine + prostigmine	2.8	6.1	8.6	10.6
0.01 M pyruvate in buffer				
O—Control (Values are average of duplicate runs)	2.4	5.1	6.7	8.0
Fumarate	4.4	9.4	13.0	15.1
Cytochrome	2.7	5.7	7.6	8.8
Prostigmine	2.7	5.9	8.3	10.9
Ephedrine	2.7	5.4	7.3	9.0

DISCUSSION

The results in these experiments differ in some respects from those obtained in similar studies previously reported.[1] In the present cases the Q_{O_2} of the unsupplemented myasthenic muscle was lower than normal and the addition of dicarboxylic acids produced a definite increase in oxygen consumption, whereas in the earlier experiments the control Q_{O_2} was normal and it was

not appreciably augmented by the addition of succinate. The difference may have been due to the fact that, in the observations reported in this paper, prostigmine and ephedrine were withheld for at least twenty-four hours before the biopsy, thus rendering the patients definitely "myasthenic," while in the previous experiments prostigmine was not withdrawn and actually was given two hours before the biopsies were performed.

In Case 1 the most interesting feature is certainly the very low respiration of the unsupplemented tissue and the rapidity with which the rate of oxygen consumption fell off after the first hour. In these respects the behavior of the muscle in vitro might be considered as exemplifying the clinical condition of the patient at the time of biopsy. Weakness was quite apparent, and an electrocardiogram showed abnormalities which were much less marked in a subsequent tracing taken after treatment with prostigmine and ephedrine had been resumed.

In Case 2, while the oxygen consumption of the unsupplemented tissue was still below normal, it was not as low as in the first case, despite the fact that the second patient was less tolerant of the withdrawal of prostigmine. This, of course, suggests that the decreased oxygen uptake of the respiring muscle is not proportional to the severity of the disease or to the clinical requirement for prostigmine. It may well be asked whether the depression in muscle respiration is reflected in a subnormal consumption of oxygen by the patient as a whole. Unfortunately, no determinations of the basal metabolism were made in Case 1 at this time, though rates several years previously when the patient was having severe symptoms had been within normal limits. Generally speaking, patients with myasthenia gravis do not have a low metabolism, and the basal metabolic rate in the second patient was –7 per cent. We have, then, a situation similar to that involving diabetes and the action of insulin,[2] in which abnormalities in tissue respiration can be demonstrated without corresponding changes in the respiratory metabolism of the intact organism. The explanation of these discrepancies must await further knowledge which may enable us better to correlate the results of in vitro studies in general with those obtained from the intact individual.

It is of interest that in Case 1, prostigmine, and in Case 2, prostigmine and ephedrine, both of which were clinically effective in our patients, significantly increased the oxygen consumption of the respiring muscle. In several experiments with normal human muscle such effects have not been observed.

The number of experiments is too small and their scope too narrow to draw any definite conclusions regarding the nature of the lesion in myasthenia gravis. It may be pointed out, however, in connection with the theory postulating a defect in the myoneural junction, that in these in vitro studies the observed effects of various supplements were most likely a result of a change in the metabolism of the muscle itself.

SUMMARY AND CONCLUSIONS

Experiments have been performed on the respiration of excised skeletal muscle from two patients with myasthenia gravis. In both patients the oxygen consumption of the muscle was lower than normal. It was increased in one by the addition of prostigmine and in the other by prostigmine and ephedrine added together. It was also increased by certain dicarboxylic acids, cytochrome C, and various combinations of these supplements. The results indicate that in this disease the affected muscles show abnormalities in intermediary metabolism.

REFERENCES

1. Stare, F. J., Gordon, E. S., and Musser, M. J.: Effect of Succinic Acid on the Respiration of Normal Human Muscle and Various Myopathies, Nature **114**: 831, 1938.
2. Ricketts, H. T., and Stare, F. J.: The Effect of Insulin on the In Vitro Respiration of Human Skeletal Muscle, J. Lab. & Clin. Med. **30**: 594, 1945.
3. Keilin, D., and Hartree, E. F.: Preparation of Pure Cytochrome C From Heart Muscle and Some of Its Properties, Proc. Roy. Soc., London, S. B **122**: 298, 1937.
4. Stare, F. J., and Baumann, C. A.: The Effect of Fumarate on Respiration, Proc. Roy. Soc., London, S. B **121**: 338, 1936.
5. Edgeworth, Harriet: A Report of Progress in the Use of Ephedrine in a Case of Myasthenia Gravis, J. A. M. A. **94**: 1136, 1930.

THE EFFECT OF INSULIN ON THE IN VITRO RESPIRATION OF HUMAN SKELETAL MUSCLE

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THE action of insulin on the respiration of muscle in vitro has been studied by a number of workers with results which may be summarized as follows:

Normal Muscle.—Insulin has been found to prolong the oxygen uptake of normal pigeon breast muscle mince respiring in various types of phosphate buffers.[1-4] Preparations of insulin rendered inert by heat or alkali are inactive in this respect.[5] As judged by the respiratory quotient, this prolonged oxygen uptake represents normal respiration.[5] This effect of insulin on pigeon breast muscle appears to be connected with the action of certain dicarboxylic acids (fumaric acid, etc.) since it is abolished by malonate which in certain concentrations likewise inhibits the catalytic action of the dicarboxylic acids.[5] In muscle from mice and frogs, Buchner and Grafe[6] found that insulin increased both the oxygen uptake and the respiratory quotient. In other experiments with normal mammalian muscle, however, namely, skeletal muscle of the dog, cat, and rabbit and cardiac muscle of the dog, insulin has been found to have no effect on the respiratory metabolism.[7]

Diabetic Muscle.—Stare and Baumann[5] reported that the stimulation by insulin of the oxygen consumption of normal pigeon breast muscle is definitely augmented by pancreatectomy, though Stadie, Zapp, and Lukens[4] did not find this effect. The addition of insulin to preparations of muscle of depancreatized dogs and cats did not result in any increase in the consumption of oxygen or any rise in the respiratory quotient.[7]

The effect of insulin on human muscle, so far as we are aware, has not been studied under these conditions. It is the purpose of this paper to report such experiments.

EXPERIMENTAL

Muscle of both diabetic and nondiabetic patients was investigated. The diabetic patients were of three types:

Group I *(Uncontrolled, insulin-sensitive)*

These patients (Cases 1-6) showed acidosis, ketosis, or marked hyperglycemia. They were all known or proved to be very sensitive to the hypoglycemic effects of insulin. The diabetes in each case was severe.

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Received for publication, April 27, 1945.

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Group II *(Controlled, insulin-sensitive)*

The single patient in this group (Case 1, formerly in Group I), was well controlled with insulin and was normally insulin-sensitive.

Group III *(Uncontrolled, insulin-insensitive)*

These patients (Cases 1, 7, and 8) were relatively resistant to insulin. Injections of the hormone were discontinued several days before biopsies were performed. The patient in Case 1 (formerly of Group I, then of Group II) had become resistant for reasons unknown. The other two patients of this group were acromegalic.

A sample of the gastrocnemius muscle weighing about 5 Gm. was removed under local anesthesia with novocain, great care being taken not to infiltrate the muscle itself. The excision was, therefore, accompanied by momentary severe pain. The muscle was freed of all obvious fatty or fibrous tissue, chilled on ice, briefly rinsed with ice water, and minced in a cold micro-latapie apparatus. The mince was collected in a cold Petri dish lined with several layers of filter paper moistened with cold physiologic saline. Samples of tissue, approximately 200 mg. each, were weighed on cellophane slips on a torsion balance. The cellophane containing the sample was placed in the manometric flask and the tissue was dispersed with a wire. Measurements of oxygen uptake were begun approximately thirty minutes after biopsy. They were carried out at a temperature of 37.5° C. for from one to four hours with and without the addition of insulin and certain other supplements, specifically fumaric and glutamic acids and, in one instance, cocarboxylase.

A glucose Ringer-phosphate buffer[8] free of calcium at pH 7.4 was used. It was made up in twice normal concentration and diluted either with water or with supplementary solutions. The phosphate concentration was M/60. The glucose content was 0.2 per cent. All supplementary solutions were made up fresh for each experiment, neutralized to pH 7.4, and used in final concentration of 0.003 M. The insulin, a commercial preparation, "Iletin" U-80, was diluted 1 to 4 with water. The flasks containing insulin received four units. The volume of solution in each flask was 2.8 c.c.

RESULTS

The data obtained from the six cases in Group I, those patients whose diabetes was severe, uncontrolled, but responsive to insulin treatment, are given in Table I. It is observed that the oxygen consumption of the unsupplemented muscle tissue was below normal in two cases (1 and 2) and in these it was raised to normal by the addition of insulin. The Qo_2 of the muscle from these two cases was 1.9 and 2.4 and in the presence of insulin was 2.8 and 3.1. The dicarboxylic acids also produced an increase in metabolism, and this was further augmented by the addition of insulin. These increases, occurring as they did during the first hour (Qo_2), represent a true stimulation of respiration, and in addition the rate of respiration was maintained for a two-hour period. The oxygen consumption of the muscle from the other four cases in this group was not increased by the addition of insulin alone. Generally the addition of a dicarboxylic acid (fumarate or glutamate) prolonged the rate of respiration and in Cases 3 and 4 this effect was observed only in the presence of insulin. It is interesting that in Cases 3 and 4 the presence of insulin alone actually produced some inhibition of oxygen uptake. In the muscle from Case 5 cocarboxylase was added to half of the vessels, and it was found that in the presence of this coenzyme insulin prolonged the rate of respiration whereas it did not do so in the absence of cocarboxylase. The experiment

TABLE I. PROTOCOLS* ON THE RESPIRATION OF MUSCLE FROM DIABETIC PATIENTS IN GROUP I (UNCONTROLLED, INSULIN-SENSITIVE)

Case 1 (Lap.), aged 54 years; severe diabetes; duration, fourteen years; marked weakness; never received insulin; blood sugar, 263 mg. per 100 c.c.; no ketosis

SUPPLEMENTS	OXYGEN UPTAKE IN Uo_2		
	1 HR.	1½ HR.	2 HR.
None (control)	1.9	3.0	3.9
Insulin	2.8	4.5	6.0
Fumarate	2.9	4.7	6.3
Insulin + fumarate	3.4	5.6	

Comment.—The control Qo_2 is low. Respiration was increased by insulin, also by fumarate, but fumarate + insulin were better than either alone.

Case 2 (Wi.), aged 16 years; severe diabetes; duration, eight months; poorly controlled with inadequate insulin; blood sugar at biopsy 550 mg. per 100 c.c.; acidosis

SUPPLEMENTS	OXYGEN UPTAKE IN Qo_2
	1 HR.
None (control)	2.4
Insulin	3.1
Fumarate	3.1
Insulin + fumarate	3.1
Glutamate	3.0
Insulin + glutamate	3.8

Comment.—Insulin, fumarate, and glutamate increased respiration about equally. Insulin + fumarate were no better than either alone, but insulin + glutamate were more effective than glutamate alone.

*All results are the average of duplicate determinations and are given as cubic millimeters of oxygen consumed per milligram of dry weight of tissue. By Qo_2 is meant the oxygen consumed during the first hour. The expression Uo_2 indicates the total amount of oxygen consumed at the end of the period designated.

Case 3 (Al.), aged 25 years; severe diabetes; duration, ten years; fair control; insulin withdrawn 5:00 P.M., December 5; on December 7 marked acidosis necessitated 80 units of insulin between 1:00 A.M. and 6:00 A.M.; biopsy 1:00 P.M., December 7, with blood sugar of 86 mg. per 100 c.c., but patient still nauseated

SUPPLEMENTS	OXYGEN UPTAKE IN Uo_2		
	1 HR.	2 HR.	3 HR.
None (control)	3.6	6.4	7.8
Insulin	3.4	5.2	5.8
Insulin added at one hour	3.4	5.3	6.0
Fumarate	4.3	6.1	6.7
Fumarate + insulin at one hour	4.0	8.6	11.6

Comment.—Insulin alone produced some depression of respiration, but when added with fumarate caused a considerable increase at two and three hours.

Case 4 (St.), aged 47 years; severe diabetes; duration, eight years; fairly good control; insulin withdrawn 8:00 A.M., March 30; marked acidosis necessitated fluids and 50 units of insulin at 11:00 P.M., March 31, and 15 units at 3:00 A.M., April 1; biopsy at 8:50 A.M. on this day with blood sugar 431 mg. per 100 c.c. and urine showing large amounts of ketone bodies

SUPPLEMENTS	OXYGEN UPTAKE IN Uo_2		
	1 HR.	2 HR.	3 HR.
None (control)	3.0	5.5	6.9
Insulin	2.6	3.6	4.0
Fumarate	3.2	6.4	7.3
Fumarate + insulin	3.4	7.2	9.0

Comment.—Insulin alone produced definite inhibition of respiration but when added with fumarate augmented the slight increase produced by the latter.

TABLE I—CONT'D

Case 5 (Eg.), aged 20 years; severe diabetes; duration, two years; poor control; insulin withdrawn March 20; biopsy March 24, with blood sugar 345 mg. per 100 c.c. and patient in ketosis; clinical and chemical acidosis four hours later

SUPPLEMENTS	OXYGEN UPTAKE IN Uo_2			
	1 HR.	2 HR.	3 HR.	4 HR.
None (control)	3.1	5.7	6.5	6.8
Cocarboxylase	2.9	5.6	7.0	7.9
Insulin	3.0	5.0	5.6	6.0
Insulin + cocarboxylase	3.0	5.8	8.6	9.5
Insulin at two hours	3.0	6.0	7.0	7.4
Cocarboxylase + insulin at two hours	3.0	5.8	8.7	9.5
Fumarate	3.5	7.4	11.2	14.1
Fumarate + cocarboxylase	3.3	7.1	10.7	13.0
Insulin + fumarate	3.1	6.3	9.5	11.6
Insulin + fumarate + cocarboxylase	3.5	6.7	10.0	13.2

Comment.—Insulin had no effect on the respiration when only the customary glucose-Ringer-phosphate buffer was used. When cocarboxylase was added, a moderate increase was produced by insulin. This was manifested chiefly as a prolongation of the original respiratory rate. Thus, if only the oxygen consumed during the last two hours of each experiment is considered, it is found that the control tissue used 7.9 – 5.6 = 2.3 c.c. of oxygen per milligram of dry weight of tissue, whereas the tissue to which insulin was added consumed 9.5 – 5.8 = 3.7 c.c., an increase of 62 per cent. Fumarate was ineffective in facilitating the action of insulin.

Case 6 (Am.), aged 21 years; moderately severe diabetes; duration, three months; never received insulin; blood sugar at biopsy, 338 mg. per 100 c.c., and patient in ketosis but not acidosis

SUPPLEMENTS	OXYGEN UPTAKE IN Uo_2			
	1 HR.	2 HR.	3 HR.	4 HR.
None (control)	3.4	6.7	8.5	9.1
Insulin	3.5	6.3	7.5	8.0
Fumarate	3.9	8.3	12.6	16.2
Fumarate + insulin	3.9	8.0	11.7	15.0
Glutamate	4.2	8.8	13.0	16.4
Glutamate + insulin	4.2	8.4	12.0	14.5

Comment.—No effect was produced by insulin, either alone or with other supplements.

with muscle from Case 6 is of interest because the respiration was unusually good. The Uo_2 even at three or four hours and the response of the muscle to fumarate and glutamate compared favorably with what one would expect with "normal" human skeletal muscle. Insulin was without effect either alone or when added to a dicarboxylic acid.

Only one experiment was done with muscle from a patient representative of Group II, that is, a controlled diabetic patient responsive to insulin therapy. The data from this experiment are given in Table II. It is observed that the unsupplemented muscle had a Qo_2 of 2.6 which is on the low side of what we have found for supposedly normal human skeletal muscle. Insulin was without effect either alone or in the presence of fumarate.

In Table III are given the results with muscle obtained from patients in Group III who were uncontrolled diabetic patients and not responsive to insulin. In these three experiments insulin produced no significant effect except in muscle from Case 8 where it definitely inhibited oxygen consumption. It is also interesting that the muscle from this last case had a very low Qo_2 and that it did not respond in the usual manner to dicarboxylic acid supplements.

The data secured in one subject (Case 1), who passed successively through all the phases of diabetes represented by Groups I, II and III, and in whom muscle biopsies were obtained during each stage, are of particular interest.

TABLE II. PROTOCOLS* ON THE RESPIRATION OF MUSCLE FROM DIABETIC PATIENTS IN GROUP II (CONTROLLED, INSULIN-SENSITIVE)

Case 1 (same as Case 1, Group I); well controlled for three weeks with from 50 to 90 units insulin per day; allergic reactions at injection sites avoided by intramuscular route; insulin not withdrawn prior to biopsy

SUPPLEMENTS	OXYGEN UPTAKE IN Uo_2	
	1 HR.	1½ HR.
None (control)	2.6	4.4
Insulin	2.7	4.5
Fumarate	3.2	5.2
Insulin + fumarate	3.4	5.7

Comment.—No appreciable effect was produced by insulin.

*All results are the average of duplicate determinations and are given as cubic millimeters of oxygen consumed per milligram of dry weight of tissue. The expression Uo_2 indicates the total amount of oxygen consumed at the end of the period designated.

TABLE III. PROTOCOLS* ON THE RESPIRATION OF MUSCLE FROM DIABETIC PATIENTS IN GROUP III (UNCONTROLLED, INSULIN-INSENSITIVE)

Case 1 (same as Case 1, Groups I and II); definitely resistant to insulin for three months, requiring from 400 to 1,200 units (average about 725 units) per day; insulin withdrawn for fifty-one hours except for 15 units (acidosis) given fifteen hours before biopsy; blood sugar, 390 mg. per 100 c.c.; moderate ketosis

SUPPLEMENTS	OXYGEN UPTAKE IN Uo_2		
	1 HR.	2 HR.	3 HR.
None (control)	2.7	4.3	4.7
Insulin	2.8	4.0	4.4
Fumarate	4.0	6.1	6.7
Insulin + fumarate	3.5	5.1	5.5

Comment.—No significant effect was produced by insulin.

Case 7 (Cr.), aged 33 years; acromegaly, four years; diabetes, two years, requiring from 150 to 300 units insulin per day; insulin withdrawn seventeen days before biopsy; blood sugar, 266 mg. per 100 c.c.; glycosuria, 4 plus; no ketosis

SUPPLEMENTS	OXYGEN UPTAKE IN Uo_2			
	1 HR.	2 HR.	3 HR.	4 HR.
None (control)	3.0	5.8	7.6	8.2
Insulin	3.2	6.0	7.4	7.7
Fumarate	3.5	7.1	9.9	11.4
Fumarate + insulin	3.7	7.9	11.0	12.5

Comment.—Insulin neither alone nor with fumarate produced any significant change in the respiration.

Case 8 (Lan.), aged 57 years; severe diabetes; duration, twelve years; control fair to good; acromegaly six years; also pyelonephritis and neuritis; for three days before biopsy, insulin dose, previously 55 units per day, reduced to 20 units per day; blood sugar at time of biopsy, 419 mg. per 100 c.c.; no ketosis

SUPPLEMENTS	OXYGEN UPTAKE IN Uo_2	
	1 HR.	2 HR.
None (control)	1.5	4.0
Insulin	1.1	2.7
Fumarate (0.007 M.)	1.9	3.2
Fumarate + insulin	1.4	3.8
Glutamate (0.007 M.)	1.6	4.1
Glutamate + insulin	1.7	4.2

Comment.—The Qo_2 is very low. Insulin alone definitely depressed respiration. Insulin with other supplements had no significant effect.

*All results are the average of duplicate determinations and are given as cubic millimeters of oxygen consumed per milligram of dry weight of tissue. By Qo_2 is meant the oxygen consumed during the first hour. The expression Uo_2 indicates the total amount of oxygen consumed at the end of the period designated.

At a time when this patient had not received treatment of any kind and when the blood sugar was high, the oxygen consumption of the unsupplemented muscle was low and was brought up to normal by the addition of insulin to the Warburg flask (Table I). After the disease had been adequately treated, the in vitro respiration of the unsupplemented tissue was higher and well sustained but was not increased by the addition of insulin to the medium (Table II). Similar results were obtained in a third experiment several months later when the patient, now highly resistant to insulin and requiring from 400 to 1,200 units per day, had lapsed from control.

It is difficult in experiments of this type to have proper controls and to be able to compare the results with similar studies done on supposedly normal skeletal muscle. For examples of the latter, we have usually used biopsies of the abdominal rectus, gluteus maximus, and gastrocnemius obtained at routine surgery from individuals without known metabolic or muscular disease. As a result of some forty experiments of this type, we have found that these samples of skeletal muscle are alike in their in vitro oxygen uptake and response to various supplements. The Qo_2 averages between 2.5 and 4.0, and a linear rate of oxygen uptake is maintained for from one and one-half to two hours. Response to dicarboxylic acids is similar to that obtained from skeletal muscle from other species,[3] and insulin is without effect on the in vitro oxygen uptake.

SUMMARY

Insulin has been found to increase and prolong the rate of in vitro oxygen consumption of minced skeletal muscle excised from severely diabetic patients whose diabetes was uncontrolled but responsive to insulin therapy. In two patients the effect was observed without the addition of other supplements; in two patients it was observed only after a dicarboxylic acid had been added to the medium; in one patient it was necessary to have added cocarboxylase; and in one patient no insulin effect either alone or in the presence of a dicarboxylic acid was found.

Insulin was without effect on the in vitro respiration of skeletal muscle from a controlled insulin-sensitive diabetic patient or from three patients whose diabetes was uncontrolled and not readily responsive to insulin therapy. Likewise, insulin gave no effect on the in vitro respiration of skeletal muscle from patients without metabolic or muscular disease.

We wish to express our appreciation to Dr. E. S. G. Barron, for the use of his laboratory and equipment, and to Drs. Alexander Brunschwig, T. Howard Clark, Dwight Clark, and Keith Grimson, for performing the biopsies.

REFERENCES

1. Krebs, H. A., and Eggleston, L. V.: The Effect of Insulin on Oxidations in Isolated Muscle Tissue, Biochem. J. **32**: 913, 1938.
2. Shorr, E., and Barker, S. B.: The In Vitro Effect of Insulin on the Respiration of Minced Mammalian Muscle, Am. J. Physiol. **126**: 629, 1939.
3. Stare, F. J., and Baumann, C. A.: Fumarate in Biological Oxidations, Cold Spring Harbor Symposia on Quantitative Biology **7**: 227, 1939.
4. Stadie, W. C., Zapp, G. A., and Lukens, F. D. W.: The Effect of Insulin Upon Oxidations of Isolated Minced Muscle Tissue, J. Biol. Chem. **132**: 411, 1940.
5. Stare, F. J., and Baumann, C. A.: The Effect of Insulin on Muscle Respiration, J. Biol. Chem. **133**: 453, 1940.
6. Buchner, S., and Grafe, E.: Studien über Gewebsatmung: I. Zur Kenntnis der Wirkungsweise des Insulins, Deutsches Arch. f. klin. Med. **144**: 67, 1924.
7. Shorr, E., and Barker, S. B.: The In Vitro Action of Insulin on Minced Avian and Mammalian Muscle, Biochem. J. **33**: 1798, 1939.
8. Stare, F. J., and Baumann, C. A.: The Effect of Fumarate on Tissue Respiration, Proc. Roy. Soc., London, S. B. **121**: 338, 1936.

THE EFFECT OF ULTRAVIOLET IRRADIATION ON THE BLOOD HEMOGLOBIN

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THE influence of ultraviolet radiation on the formation of hemoglobin and erythrocytes and its effectiveness in the treatment of anemia have not been clearly established. The literature reveals much conflicting evidence with few controlled clinical experiments bearing on the subject. It has been shown that repeated irradiation to animals stimulates hematopoiesis,[1, 2] causing an unusually rapid production of erythrocytes. Mayerson and Laurens have shown that irradiation with the flaming carbon arc and the mercury quartz lamp produces a marked increase in the erythrocytes and reticulocytes in dogs made anemic by hemorrhage[3] as well as in dogs in which a hemolytic anemia was produced by the administration of acetylphenylhydrazine (pyrodine).[4] Ultraviolet irradiation has also been reported to be effective in raising the hemoglobin level in rats rendered anemic by a milk diet.[5]

There have been many reports concerning the effect of ultraviolet irradiation on the anemia of human subjects. Kovacs[6] observed its effect on fifty-nine patients whose anemia was secondary to a wide variety of diseases. He noted a slight increase in the blood hemoglobin in fifteen of the thirty-seven patients treated and an increase in the erythrocytes in seventeen patients. The effects were not striking. In a subsequent report[7] he concluded that the production of hemoglobin is accelerated and the number of erythrocytes is increased by ultraviolet irradiation. It has been shown that this form of therapy causes an increase in the blood hemoglobin in debilitated children,[8] but Laurens[9] and Krusen[10, 11] believe that although the radiation may have some effect on certain forms of anemia, its usefulness is limited, its effects are nonspecific, and it serves only as a useful adjunct to other forms of treatment. Ultraviolet irradiation is of proved value in the treatment of certain conditions such as rickets, bone and joint tuberculosis, and some other disease which may be accompanied by an anemia. As the primary disease is improved by this treatment, there is a concomitant improvement in the anemia.

In an attempt to evaluate the effect of ultraviolet irradiation on hemoglobin formation, we selected forty subjects with mild, but uncomplicated, anemia. These patients were students and hospital personnel who were in good health and whose history and physical examination revealed no recognizable disease except for the low blood hemoglobin. We have encountered many similar subjects whose hemoglobin level was below the commonly accepted standards of normal but in whom there was no recognizable cause for the anemia. It has been our belief[12] that this low level of hemoglobin represents a normal level for some individuals, whereas in others a similar hemoglobin level represents a mild grade of anemia which, in the absence of other causative factors, may be on a nutritional basis. The administration of iron salts to such subjects produces an increase in their hemoglobin level which reaches a peak at the end of from eight

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Received for publication April 14, 1945.

to ten weeks and then falls. The blood hemoglobin may drop to the pre-treatment level in the nonanemic subjects or to a point slightly above this level in those with mild anemia. This type of subject was selected for irradiation therapy in the belief that any stimulating effect which the rays might have would become evident in as much as there were no complicating diseases or deficiencies in the individuals and the bone marrow was apparently normal. Iron salts had previously been administered to many of the women and to some of the men subjects without significant or persistent benefit. All the subjects ate an adequate and well-balanced diet, but by reason of their occupation they were indoors much of the time without exposure to sunshine.

These observations were made during the fall and winter months of the year when there is a tendency, in this locality, for a slight but gradual decrease in the blood hemoglobin. As a control, a group of thirty-two subjects (twenty-five women and seven men) were selected whose hemoglobin levels corresponded to those of the group under treatment. The control subjects were observed during the same months as the group under treatment, their environment and eating habits were essentially the same, and they received no medication or treatments.

The radiation used in this study was obtained from a Burdick air-cooled quartz lamp. The burner of the lamp was placed thirty inches above the body

TABLE I

SUBJECT	INITIAL BLOOD HEMOGLOBIN GRAMS	WEEKLY BLOOD HEMOGLOBIN (GM.) I	II	III	IV	V	VI	VII	VIII	IX	X
Men											
J. D.	12.77	+1.86	+1.36	+ .87							
R. P.	12.34	- .19	+ .81	- .06	+1.05	+1.05					
E. P.	12.34	-	-1.05	+ .19	- .31	-	+ .56				
E. A.	12.77	+ .12	+1.12	+ .12	± .00	+ .74	- .56				
R. B.	13.89	+ .12	+ .50	+ .50	+ .99	-	- .74	+ .12			
P. B.	13.39	- .25	- .12	+ .25	+ .37	-	+ .37	-	+1.98		
D. M.	13.89	+ .50	+1.12	+ .75	+ .99	-	-	+ .25	+1.49	+1.98	
L. S.	12.77	- .37	- .12	-	+ .87	- .12	+ .75	+ .25	-	-	+ .62
R. A.	13.64	+1.36	- .50	+ .50	-	+ .99	+ .62	+ .62	± .00	- .99	- .83
G. H.	12.90	+ .12	+1.61	+1.24	+ .50	+1.12	- .62	- .87	- .76	+ .99	-
S. S.	12.90	+1.86	+ .62	+ .74	+1.49	-	+2.73	+2.23	+ .87	+ .99	+ .62
J. N.	12.09	+1.80	-	+ .68	+ .68	+ .93	-	-	-	-	+1.55
O. L.	12.77	-	+1.24	± .00	-	+ .87	+1.61	- .25	+ .50	+ .50	+1.24
Women											
J. W.	9.77	-	+ .30								
V. T.	9.17	+ .90	-	+1.77							
H. B.	12.21	+ .81	+ .56	- .25	- .81						
J. D.	12.40	- .74	- .50	-	+ .12						
P. C.	9.36	-	-	-	-	-	+ .75				
R. K.	11.28	-	- .31	-	- .31	-	- .62	+ .19			
P. L.	9.80	-	+ .68	-	+ .68	+1.24	+ .56	+ .31	+ .43		
M. P.	9.92	-	-	+ .50	-	+ .43	-	-	-	+ .81	
A. D.	10.04	+ .93	+ .88	-	-	-	-	-	+ .42	+1.12	
M. C.	10.23	+ .12	+1.12	+ .12	+ .81	+ .31	+ .93	+ .31	+ .43	+1.12	
L. G.	10.35	± .00	+1.24	-	-	+ .06	+ .50	-	- .43	- .12	
B. H.	11.72	+ .62	+ .68	+ .46	+ .93	+ .31	+ .93	+1.18	-	+1.05	
W. B.	10.66	+ .62	-	+ .25	-	+2.73	+1.36	+ .93	-	-	+ .50
G. G.	9.24	-	-	+ .68	+ .93	-	-	+ .12	- .68	-	-
E. H.	10.66	+ .37	-	+1.36	-	+ .99	-	-	-	-	+ .99
R. K.	12.77	+ .37	+ .37	- .56	-1.43	+1.24	-	-	-1.18	-1.24	-
F. C.	11.04	- .56	+ .93	-	+ .74	± .00	- .06	+ .12	+1.36	+1.05	-
E. Z.	11.47	+1.18	-	+ .56	+ .81	+1.55	-	-	+1.30	+1.05	+2.29
M. W.	11.72	-	+1.55	+ .56	- .37	+ .50	+1.30	+ .93	+ .06	+ .62	+ .81
E. Y.	12.40	± .00	-	- .62	-	± .00	-	- .12	+ .87	-	-
L. C.	12.52	-	± .00	+ .87	- .12	± .75	+ .25	+ .62	- .66	+ .19	- .43
L. M.	12.65	- .93	- .56	-1.55	- .62	- .43	- .50	+ .62	+ .74	+1.05	-1.47
E. C.	12.09	- .56	- .87	- .93	-1.67	- .81	- .87	-	± .00	-	-
E. J.	10.85	+ .93	-	-	+ .06	-	- .31	-	- .31	± .00	- .31
E. H.	10.48	+ .31	+ .43	+ .62	+ .99	+ .50	+ .87	-	+ .68	-	-
M. P.	10.54	+ .31	-	-1.12	-	- .12	-	-	- .87	-	+ .19
E. H.	9.02	-	+ .31	-	+ .90	-	+1.20	-	-	+1.35	-

and the shutters were adjusted so as to concentrate the light on the exposed portion of the body. The erythema dose was determined for each individual, being about thirty seconds in most cases, and this amount of irradiation was given as the initial exposure on both the anterior and posterior surfaces of the body, respectively. Barring reactions, the amount of irradiation was increased thirty seconds with each of the three treatments per week until a maximum exposure of seven minutes on both the ventral and dorsal surfaces was reached. This exposure was given for one week, then reduced to thirty seconds, and again built up in the same manner. In the few instances in which a significant erythema developed, the exposure time was decreased thirty seconds, held at that level for several treatments, and then increased in the usual manner.

Blood hemoglobin determinations were made at weekly intervals, using an acid hematin method with a Klett-Summerson photoelectric colorimeter.

The results of the weekly blood hemoglobin determinations are shown in Table I and the change in the hemoglobin levels for the treated and the control groups are illustrated graphically in Fig. 1.

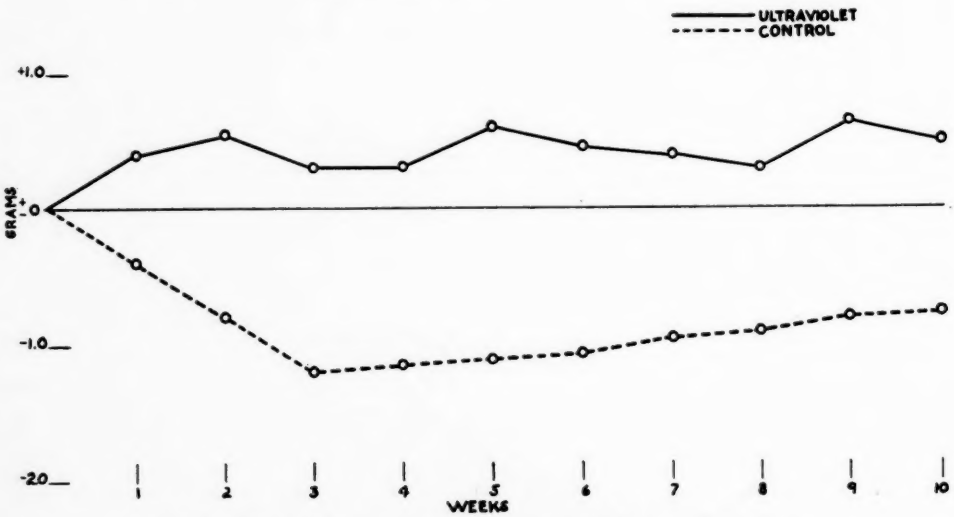

Fig. 1.—Effect of ultraviolet irradiation on blood hemoglobin level.

It will be noted in Table I that a majority of the subjects, both men and women, who received ultraviolet irradiation showed a rise in blood hemoglobin. This was not a striking increase, but only a moderate elevation in all cases. The increase in hemoglobin is portrayed graphically in Fig. 1 as the average increase for the group. It will be noted that although there is a slight increase in the hemoglobin level which is maintained throughout the observation period, there is not a progressive and continuing rise. Hemoglobin readings made at weekly intervals on any group of individuals will show variations which are as great as the variations encountered in this group of subjects, but they will not show the persistent increase. The results assume greater significance when compared to the control group and when it is realized that the observations were made during a time of year when there is a normal tendency in this locality for a slight reduction in blood hemoglobin.

CONCLUSION

These results indicate that ultraviolet irradiation produces a very slight but persistent increase in the blood hemoglobin level. The increase is not great

enough to recommend this as a routine therapeutic procedure in hypochromic anemia, although it may sometimes be useful as a supplement to other forms of therapy in selected cases.

The Burdick Corporation of Milton, Wis., kindly furnished the Quartz Mercury Ultraviolet lamp used in this study, a Burdick Professional Special.

REFERENCES

1. Laurens, H.: The Physiological Effects of Radiation, Physiol. Rev. **8**: 1, 1928.
2. Mayerson, H. S., and Laurens, H.: The Effects of Carbon Arc Radiation on the Blood of Dogs, Am. J. Physiol. **86**: 1, 1928.
3. Laurens, H., and Mayerson, H. S.: The Effects of Radiant Energy on Hemorrhagic Anemia, J. Nutrition **3**: 465, 1931.
4. Mayerson, H. S., and Laurens, H.: The Effects of Radiant Energy on Experimental Hemolytic Anemia, J. Nutrition **4**: 351, 1931.
5. Foster, P. C.: The Effects of Radiant Energy on Milk Anemia in Rats, J. Nutrition **4**: 517, 1931.
6. Kovacs, R.: Ultraviolet Irradiation for Secondary Anemia, New York State J. Med. **35**: 825, 1935.
7. Kovacs, R.: Ultraviolet Irradiation in Secondary Anemia, Arch. Phys. Therapy **18**: 756, 1937.
8. Morton, Eva: Value of Ultraviolet Light in Industrial Districts, Brit. Jour. Phys. Med. **12**: 117, 1937.
9. Laurens, H.: The Physiologic Effects of Ultraviolet Radiation, J. A. M. A. **111**: 2385, 1938.
10. Krusen, F. H.: Medical Application of Ultraviolet Radiant Energy, Ann. Int. Med. **14**: 641, 1940.
11. Krusen, F. H.: Present Status of Ultraviolet Irradiation, Arch. Phys. Therapy **22**: 199, 1941.
12. Fowler, W. M., and Barer, A. P.: Some Effects of Iron on Hemoglobin Formation, Am. J. M. Sc. **201**: 642, 1941.

THE EFFECT OF DIGITALIS, EPINEPHRINE, AND SURGERY ON THE RESPONSE TO HEPARIN

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THE current interest in anticoagulants in the prevention of thrombosis has led to renewed discussion of the effects of various agents on the coagulation time.[1-5] Recently de Takats[6] has described a heparin tolerance test for use in the study of the coagulation mechanism and has commented on the effect of various agents on the response to heparin.[1, 4-6] This study was carried out to determine in our own experience the effects of epinephrine, digitalis, and major surgery on the heparin tolerance test.

METHODS

In measuring heparin tolerance it is of prime importance that the coagulation time be followed by a method capable of accurately revealing small changes. The methods of Howell and of Lee-White, while suitable for many clinical purposes, do not detect relatively small changes in the coagulation

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The patients used in this study were obtained through the courtesy of Dr. A. H. Colwell and the attending staff of The Presbyterian and Woman's Hospitals of the University of Pittsburgh.

The author is indebted to Dr. T. K. Kruse and Dr. C. C. Guthrie for much helpful criticism.

Received for publication, May 3, 1945.

time[7] and therefore were not used in this study. Kruse's method, which indicates the initiation of coagulation as contrasted to the relatively complete coagulation obtained with the Lee-White procedure, was found in preliminary work to be a satisfactorily sensitive test of the coagulation time.[7]

After control coagulation times had been established, a dose of sodium heparin of 0.15 mg. per kilogram body weight was administered intravenously. The coagulation time was then determined at 2- to 5-minute intervals until it had returned to control levels. A fresh skin puncture was used for each determination.

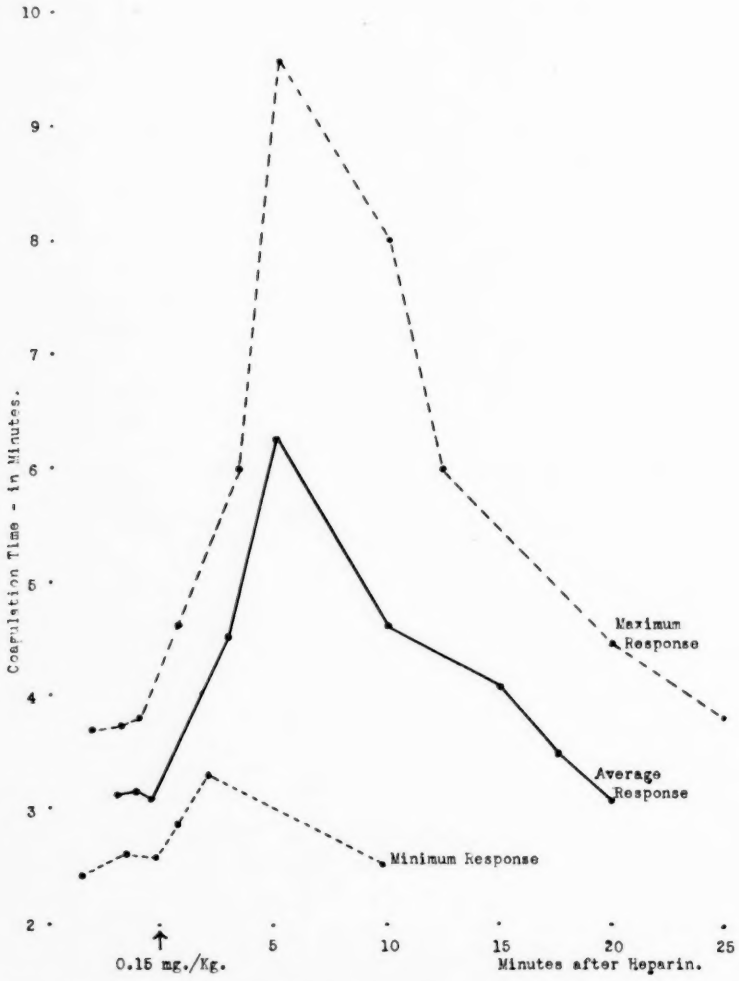

Fig. 1.—Average, maximum, and minimum response to 0.15 mg. heparin per kilogram of body weight.

RESULTS

Control Experiments.—Control coagulation times in the twenty-five individuals tested varied from 2.5 to 5 minutes. Following the administration of 0.15 mg. of heparin per kilogram of body weight the coagulation time rose from 1 to 8 minutes above the control level within 10 minutes after injection. In all control individuals the coagulation time had returned to the base level within 25 minutes. In Fig. 1 is indicated an average response to this dose of heparin as well as the maximum and minimum increase noted in this series. De Takats[6] has previously commented on hypo- and hyperreactors to heparin and de-

scribed a biphasic reaction[1] with a primary and secondary peak in coagulation time. Such biphasic reactions were not observed in this series. Repetition of the tests even after several weeks revealed relatively constant responses. This is demonstrated in Fig. 2.

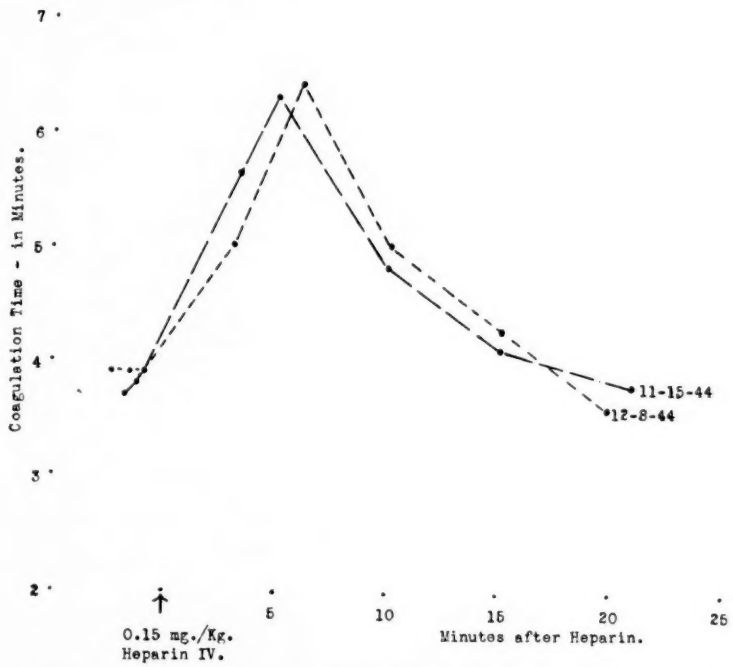

Fig. 2.—Consistent response to administration of 0.15 mg. sodium heparin per kilogram of body weight repeated after three-week interval.

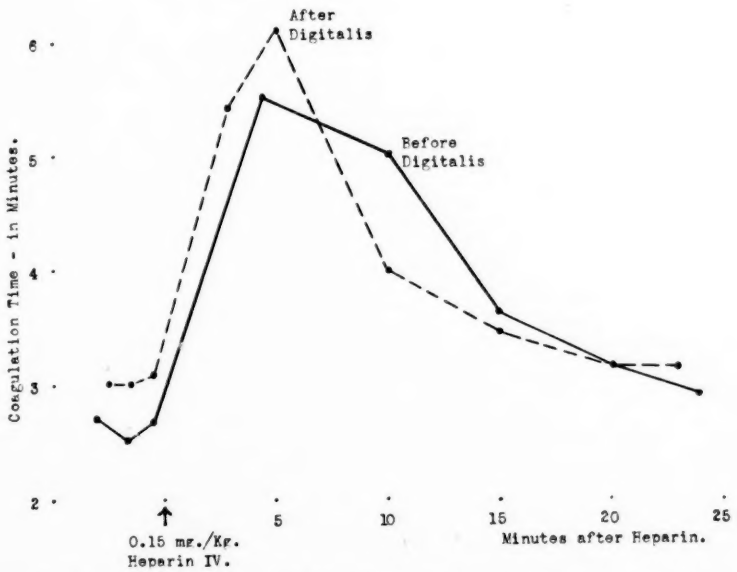

Fig. 3.—Response to heparin before and after the administration of 141 gr. of digitalis in thirty-three days.

Digitalis Administration.—Several investigators[2-5] have found a reduction in the coagulation time following the administration of digitalis. This has been reported to be especially evident with the giving of relatively large doses to animals.[2-4] With the use of therapeutic amounts in man[5] the decrease in

coagulation time as compared to the controls was much less evident. The heparin tolerance of eight individuals before, during, and after therapeutic digitalization failed to reveal any significant or consistent decrease in either the coagulation time or the response to heparin in this study. In Fig. 3 is illustrated the response to heparin before and after the administration of 141 gr. of digitalis in thirty-three days.

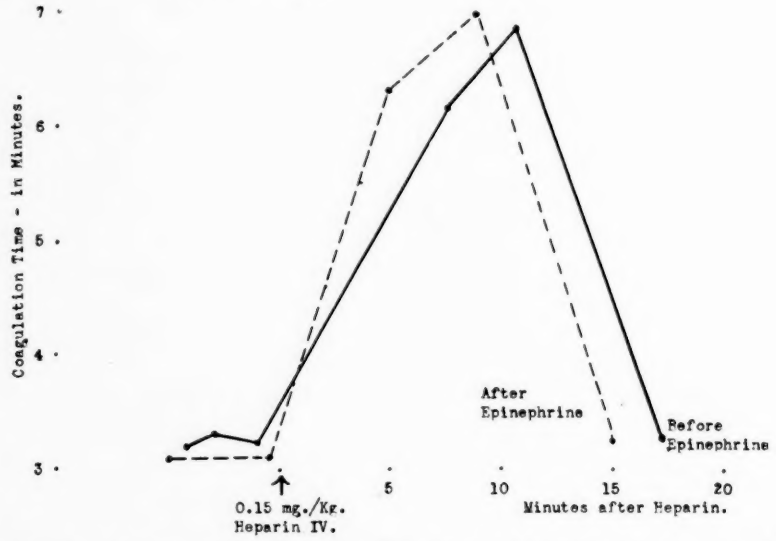

Fig. 4.—Heparin tolerance curve before and after the administration of 1 mg. of epinephrine intravenously.

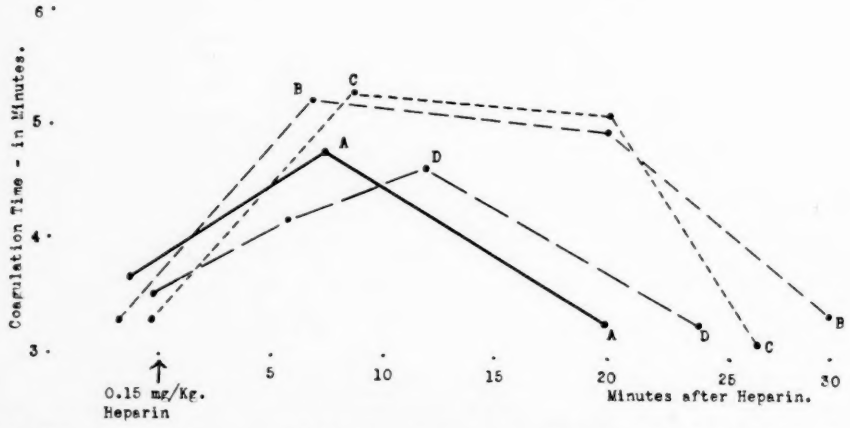

Fig. 5.—Effect of heparin on coagulation time before, *A*, and three days, *B*, five days, *C*, and seven days, *D*, after subtotal gastrectomy.

Epinephrine.—Epinephrine reportedly hastens coagulation of blood but such reports have not been generally confirmed.[8] Recently a decrease in the heparin tolerance curve has been reported following the administration of epinephrine.[1] In five individuals no significant or consistent change in the heparin tolerance curve following the administration of 1 mg. of epinephrine intravenously has been obtained in this study. In Fig. 4 is illustrated a typical response.

Surgery.—It is generally recognized that thrombosis is prone to occur in the postoperative period. In nine patients in whom the coagulation time and heparin tolerance was followed before and at intervals after a major surgical

procedure, no significant or consistent decrease has been obtained. In Fig. 5 are indicated the results of the heparin tolerance test before and at three, five, and seven days after subtotal gastrectomy. In this individual an actual prolongation of the heparin tolerance curve was obtained rather than a decrease as observed by others.[6]

SUMMARY

A study of the response of individuals to the intravenous administration of 0.15 mg. of heparin per kilogram of body weight is presented.

No significant decrease in heparin tolerance was obtained after the administration of digitalis or epinephrine or after major surgery.

REFERENCES

1. de Takats, G.: Nervous Regulation of the Clotting Mechanism, Arch. Surg. **48**: 105, 1944.
2. Macht, D. I.: Experimental Studies on Heparin and Its Influence on Toxicity of Digitaloids, Congo Red, Cobra Venom, and Other Drugs, Ann. Int. Med. **18**: 772, 1943.
3. Werch, S. C.: Reduction of the Coagulation Time of Rabbits Blood by Digitalis, Quart. Bull. Northwestern Univ. M. School **17**: 50, 1943.
4. de Takats, G. Trump, R. A., and Gilbert, N. C.: The Effect of Digitalis on the Clotting Mechanism, J. A. M. A. **125**: 840, 1944.
5. Massie, E., Stillerman, H. S., Wright, C. S., and Minnich, V.: Effect of Administration of Digitalis on Coagulability of Human Blood, Arch. Int. Med. **74**: 172, 1944.
6. de Takats, G.: Heparin Tolerance, Surg., Gynec. & Obst. **77**: 31, 1943.
7. Kruse, T. K., and Moses, C.: A Method for Determining the Coagulation Time Using Capillary Blood, J. Lab. & Clin. Med. **30**: 631, 1945.
8. Wiggers, C. J.: Physiology in Health and Disease, ed. 4., Philadelphia, 1944, Lea & Febiger, p. 345.

LABORATORY METHODS

A DEMAND APPARATUS FOR AUTOMATIC DELIVERY OF AEROSOLS DURING INSPIRATION

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THE nebulization of bronchodilator and chemotherapeutic aerosols by a continuous stream of oxygen passing through a nebulizer delivers these drugs during both cycles of respiration, thus permitting a loss of the solution during the expiratory cycle.[1, 2] In the clinical use of penicillin aerosol a Y tube was inserted between the regulator and the nebulizer. The open end of the Y tube was closed by application of a finger just before the beginning of inspiration, which forced the oxygen flow of from 4 to 8 liters per minute through the nebulizer, and, before the termination of inspiration, the finger was removed, which permitted the oxygen stream to pass into the outside atmosphere and terminate the nebulization of the penicillin. The aerosol was thereby produced and inhaled during inspiration only. The volume of the nebulizer was enlarged to contain a 1,000 c.c. reservoir bulb to collect penicillin in the exhaled air.[3, 4]

In the apparatus here described the lowering of pressure within the nebulizer at the start of inspiration releases a flow of oxygen from the demand valve which then passes through the nebulizer. The penicillin aerosol is thus formed only during inspiration without the cooperation of the patient being required.

DESCRIPTION

The automatic nebulizing demand valve (Fig. 1) consists of an aluminum casing divided into three separate chambers (*1*, *2*, and *3*). The first chamber, *1*, houses the valve, *B*, which controls the flow of gas to the nebulizer. The second chamber, *2*, is kept at atmospheric pressure by means of two holes communicating with the outside air. It is separated from the third, or activating chamber, *3*, by a thin rubber diaphragm, *E*, which is stiffened with two thin sheets of plastic, *D*. A valve stem, *C*, connected to the rubber diaphragm, and passing through a bearing into the top chamber, *1*, is capped with a head containing a neoprene washer. The valve stem is raised and lowered by movement of the rubber diaphragm and when fully elevated its head makes contact with the valve seat, *B*, interrupting the flow of gas which flows through the outlet, *J*. An ordinary hospital regulator attached to the demand valve by means of the adapter nut, *A*, supplies the air or oxygen flow for the system.

The third, or activating chamber, *3*, contains a lever arm, *F*, which is pressed upward against the underside of the diaphragm by means of a spring, *G*. The spring, housed in a gastight cap, may be shortened or lengthened to exert varying degrees of tension against the lever arm. The change in spring tension

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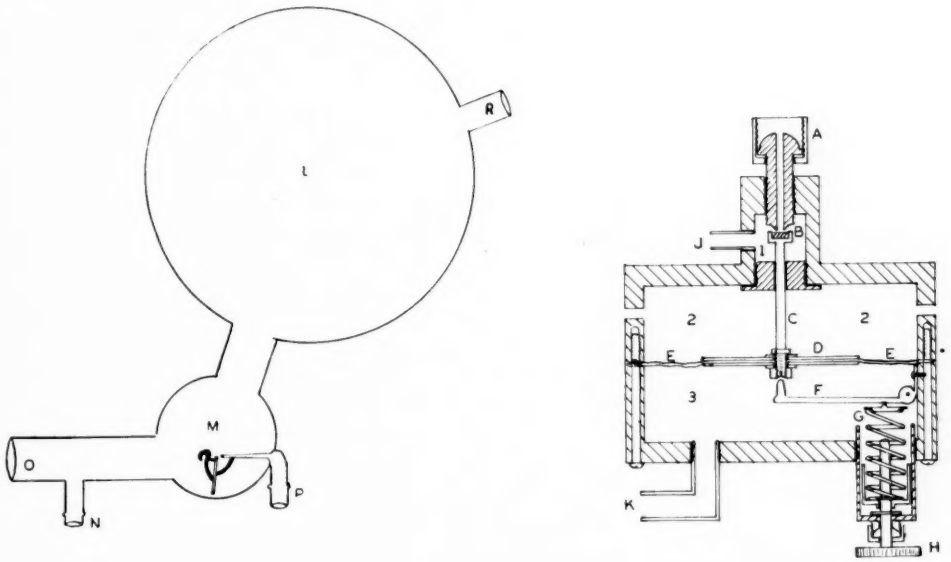
The work described in this paper was done under a contract, recommended by the Committee on Medical Research, between the Office of Scientific Research and Development and Columbia University, New York City.

Received for publication, March 17, 1945.

is accomplished by turning the thumbscrew, *H*. The outlet, *K*, from the activating chamber permits the transmission of pressure fluctuations to the underside of the diaphragm.

The nebulizer* is adapted from a type used for administration of 1 per cent epinephrin. A 500 or 1,000 c.c. flask, *L*, is fused to the top of the reservoir, *M*. As air or oxygen flows through the inlet, *P*, it converts the inhalant solution to a very fine suspension of droplets which leave the system through the mouthpiece, *O*. The velocity of gas flow through the reservoir sets up a slight negative pressure and more air is drawn from the outside atmosphere through the restricted orifice, *R*. This latter fraction of air then mixes with the suspension of droplets in *L* and *M* and leaves the nebulizer at the mouthpiece. With this arrangement any droplets of the inhalant remaining in the flask after expiration are drawn into the lung, with the newly formed aerosol, during the succeeding inspiration.



NEBULIZER AUTOMATIC NEBULIZING VALVE

Fig. 1.

Outlet *K* of the demand valve is attached to the nebulizer, *N*, by means of a three-foot length of ⅜ inch I.D. tubing. The flow outlet *J* is connected to the inlet *P* of the nebulizer with a three-foot length of ¼ inch I.D. pressure tubing.

DIRECTION FOR USE

Connect the demand regulator to a hospital flow regulator by means of the adapter nut A. Decrease the tension of the spring by turning the thumbscrew clockwise until it stops. Turn handle of the hospital regulator to deliver the flow of air or oxygen desired. This flow may be from 4 to 10 liters per minute, depending on the speed of aerosolization or density of aerosol desired. Increase the spring tension against the lower arm by turning the thumbscrew counterclockwise, until the pressure of the lever arm against the diaphragm is just

*Vaponefrin Co., Upper Darby, Pa.

sufficient to hold the valve stem against the valve seat with sufficient force to cut off the flow of gas from the regulator.

Connect the demand regulator outlets, *J* and *K*, to the nebulizer outlets, *P* and *N*, with the proper tubing. Place 1 or 2 c.c. of the inhalant solution at the bottom of the nebulizer reservoir. Direct the mouthpiece back into the mouth and have the subject hold it firmly with the lips and breathe only through the mouth. As inspiration begins there is a slight fall (1 cm. H_2O) in pressure in the flask due to the restricted intake orifice, *R*. This pressure fall is transmitted through the pressure outlets *N* and *K* and is applied to the underside of the rubber diaphragm. Since the upper side of the diaphragm is maintained at atmospheric pressure, the diaphragm will be lowered. In so doing it moves the valve stem downward and permits gas from the hospital regulator to pass through *B* and then chamber *1* and then proceed to the nebulizer inlet, *P*. Aerosolization then takes place and a mixture of the aerosol, and air entering the system through *R*, is drawn into the lungs. At the end of inspiration the pressure in the nebulizer returns to atmospheric, and this pressure transmitted to the activating chamber permits the diaphragm to rise. The rising diaphragm forces the valve stem against the seat and interrupts the flow of gas through the demand valve. During expiration the expired gas passes through the nebulizer reservoir and flask and leaves the outlet *R*. Some of the finely suspended droplets of aerosol are precipitated on the walls of the flask, some leave through *R*, and the remainder stay in suspension in the flask. The latter are then drawn into the lungs, during the succeeding inspiration.

SUMMARY

A demand regulator for nebulization of chemotherapeutic aerosols is described which automatically operates during inspiration only. This apparatus has been used for clinical administration of penicillin.

REFERENCES

1. Richards, D. W., Jr., Barach, A. L., and Cromwell, H. A.: Use of Vaporized Bronchodilator Solutions in Asthma and Emphysema, Am. J. M. Sc. **199**: 225, 1940.
2. Barach, A. L.: Principles and Practices of Inhalational Therapy, Philadelphia, 1944, J. B. Lippincott Co.
3. Barach, A. L., Silberstein, F. H., Oppenheimer, E. T., Hunter, T., and Soroka, M.: Inhalation of Penicillin Aerosol in Patients With Bronchial Asthma, Bronchiectasis, Chronic Bronchitis and Lung Abscess, Ann. Int. Med. In press.
4. Barach, A. L., Silberstein, F. H., Oppenheimer, E. T., Hunter, T., and Soroka, M.: Inhalation of Penicillin Aerosol, Proc. Soc. Exper. Biol. & Med. In press.

DETERMINATION OF HEMOGLOBIN

The Use of a Buffer as the Laking Fluid to Overcome Errors Inherent in the Carbonate Method

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THE rapid yet accurate determination of hemoglobin has for many years been a major clinical laboratory problem. With the advent of photoelectric colorimeters several of the original difficulties were overcome. In this paper, data will be presented which point out a possible error in one of the photometric methods now in general use.

The use of dilute carbonate (0.1 per cent) as the laking fluid for red cells and solvent for hemoglobin dates back to the work of Hüfner[1] and others in the latter half of 1800. In 1929 Sheard and Sanford[2] developed a relatively simple and inexpensive filter photometer primarily for the determination of hemoglobin and recommended the dilution of 0.1 c.c. of blood in 20 c.c. of 0.1 per cent sodium carbonate. Sheard and Sanford, and later workers who have used this method on other instruments, have emphasized the importance of laking and of shaking to insure oxygenation of the hemoglobin but have not discussed a critical time for the reading of the sample. In the photometric method, the light absorptive power of oxyhemoglobin at one of the maxima of absorption (542 mu) is used as a measure of the amount of pigment present.

It is generally implied that the photometric measurements be made as soon as laking and oxygenation are complete, but in a hospital laboratory where many hemoglobin determinations are made each day, the actual reading of the samples in the photelometer may be delayed.

That the time of reading of the diluted sample is important became apparent to us when we attempted to standardize several makes of photoelectric instruments. It was found on certain occasions that the light absorptive power of the solutions decreased on standing at room temperature. In Table I is shown the variation in hemoglobin values of two blood samples by the carbonate method, the diluted blood being kept at room temperature.

Table I. Decrease in Hemoglobin Values of Blood Samples Diluted 1:200 in 0.1 Per Cent Carbonate and Kept at Room Temperature

Time in minutes	Grams hemoglobin* per 100 c.c. blood	
	Sample 1	Sample 2
2	14.0	13.0
12	13.0	12.3
20	12.8	11.6
32	12.1	11.2
37	11.6	10.8
47	11.1	10.5

*Hemoglobin values were read from a standard curve used in connection with a Coleman Model 6. Transmission values were read with the instrument set at 540 mu. The standard curve relating oxyhemoglobin concentration to transmission values was prepared from suitable dilutions of blood standardized with a Van Slyke manometric apparatus.

Other samples of blood were studied in this manner and it was found that some showed greater variation, while some were quite stable. The principal variable in this work appeared to be temperature.

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Received for publication, March 30, 1945.

To test this possibility, a blood after dilution in carbonate, which had been stabe for 90 minutes at room temperature (26° C.), was immersed in a water bath at 40° C. In 15 minutes the hemoglobin value dropped 0.9 Gm. and after 30 minutes, 2.0 Gm. per 100 c.c. blood. Although 40° C. is above our laboratory room temperature, we found that during the course of our work the temperature had varied from 26 to 37° C. The greatest instability in oxyhemoglobin values was found to have occurred on the days when the temperature had been 37° C. Although these studies had been made on the Coleman Junior Clinical Spectrophotometer Model 6,* the same phenomenon (oxyhemoglobin instability at 40° C.) is easily demonstrated on filter photometers.

It was observed that as the oxyhemoglobin solutions decreased in light absorptive capacity (measured at 540 mu), the solutions changed from the bright red color characteristic of oxyhemoglobin to a dull brownish-red color. This color change was investigated spectrophotometrically, by determining the absorption curves of the pigments present in a blood diluted with carbonate and kept at 40° C.

The oxyhemoglobin content of the blood sample by the Van Slyke manometric method was 14.25 Gm. per 100 c.c. A Cenco-Sheard spectrophotelometer with 1 cm. cells was used for these studies, the entrance slit being 1 mm. and the exit slit 2.5 mu.

Dilution of the blood was accomplished by adding 0.1 c.c. blood to 20 c.c. freshly prepared 0.1 per cent sodium carbonate† in a 50 c.c. flask. The flask was stoppered, shaken, and then immersed to one-half its depth in a constant temperature water bath at 40° C. Samples of the diluted blood were withdrawn at intervals, cooled to 26° C. and absorption data determined in the Cenco instrument. In Fig. 1 are shown the absorption curves of the pigments present at 0, 60, and 180 minutes and at 24 hours.

The 0-hour curve shows the two, well-defined maxima of oxyhemoglobin at 541 to 542 mu and 576 mu. The 1-hour curve indicates that a considerable change has occurred in the oxyhemoglobin molecule, for K at 576 mu decreased from 0.625 to 0.369, and the K value for the 541 mu maximum dropped from 0.595 to 0.430. The absorption curve of the 3-hour sample shows a rather broad maximum in the 540 mu region, but the 576 maximum has disappeared. The 24-hour absorption curve shows the complete disappearance of specific absorption maxima in the 540 to 576 region.

Since it was apparent that the oxyhemoglobin was being converted to a pigment (or pigments) with different absorption characteristics, an attempt was made to determine the nature of such substances.

After the 0-hour sample had been used to obtain the data for the curve on Fig. 1, it was treated with a few small crystals of $K_3Fe(CN)_6$[3, 4] to convert the hemoglobin to methemoglobin. The absorption curve of this material is also shown in Fig. 1. Since the absorption spectra of methemoglobin vary greatly with changes in hydrogen-ion concentration,[1] it is necessary to control the pH value. We believe that by converting the oxyhemoglobin in the 0-hour sample to methemoglobin, while other factors are held constant, it is then possible to use such a system for purposes of comparison. A comparison of the curve at 1 hour with the methemoglobin curve suggests that at this time there has been a conversion of most of the oxyhemoglobin to methemoglobin. This is suggested by the lowering of the 540 mu maximum, and by a still greater lowering of the 576 mu maximum, to values approximating those of pure methemoglobin.

*Coleman Electric Co., Maywood, Ill.

†Mallinckrodt's analytical reagent grade sodium carbonate was used throughout this work.

The absorption curve of the pigments at 3 hours, on the other hand, indicates that during this period another change has occurred. The maximum at 540 to 542 mu has been lowered below that of the known methemoglobin and has been shifted to the left to the 535 to 540 mu region. The flat curve of the 24-hour sample indicates that a pigment (or pigments) other than oxyhemoglobin and methemoglobin is present. The shape of this curve is somewhat similar to that of hematin in sodium hydroxide.[1, p. 122]

Assuming that the chief pigment in the solution at 3 hours was methemoglobin, the presence in this solution of small amounts of the pigment responsible for the low, flat absorption curve of the sample at 24 hours may explain the difference between the curve of the 3-hour sample and the methemoglobin curve.

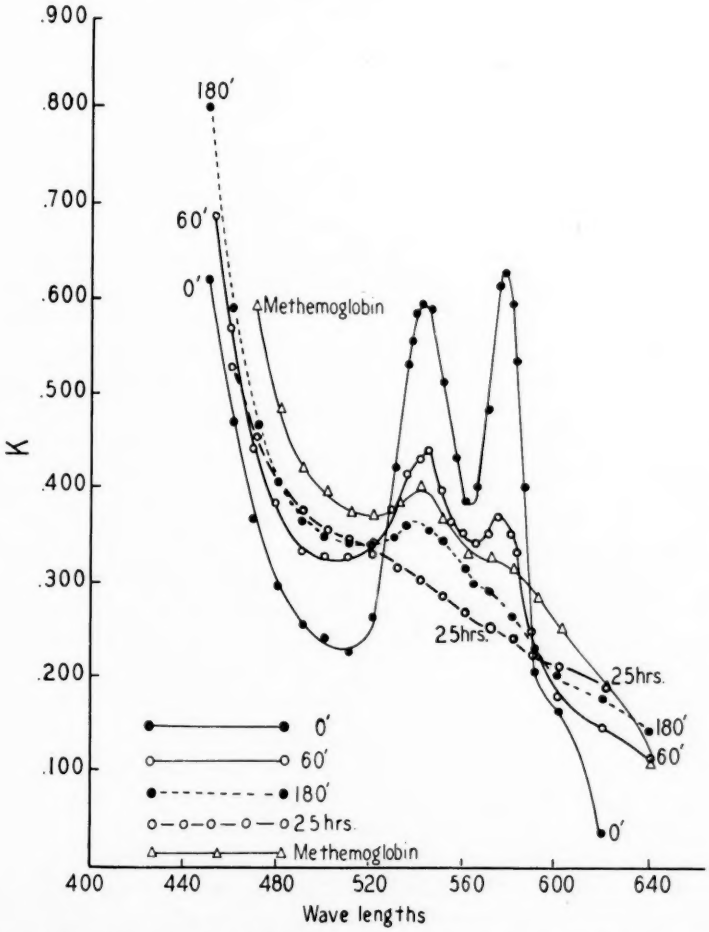

Fig. 1.—Absorption curves of pigments present in a solution of blood diluted 1:200 in 0.1 per cent sodium carbonate and maintained at 40° C. for the indicated periods of time. The ordinate, *K*, is defined as: $K = \frac{d}{1} = -\frac{\log_{10} T}{1}$. A Cenco-Sheard spectrophotelometer with 1 cm. cells was used; the entrance slit was 1.0 mm. and the exit slit 2.5 mu.

Heilmeyer[1] and Martini and Schuler[5] pointed out the swift spontaneous conversion of oxyhemoglobin to methemoglobin. The latter workers showed that irradiation, especially by short wave lengths, accelerated this conversion. Drabkin and Austin[4] found that blood diluted 1:100 in 0.1N NH_4OH showed some change upon standing at 25° C. but at a 1:1000 dilution in this solvent, absorption values at 540 mu decreased to one-half in 200 minutes. However, these authors showed that if 5 mm. phosphate at a pH of 7.4 was used as the solvent,

the oxyhemoglobin remained quite constant as measured by absorption values at 540, 560, and 575 mu.

From our observations and those of Drabkin, it appeared that the high pH (10.5) of the carbonate solvent, the increased temperature (40° C.), and the dilution of 1:200 all contributed to the instability of the oxyhemoglobin solution.

Since it is difficult to maintain a constant temperature bath at 25 to 28° C. throughout the year, we felt that adequate control of pH might enhance the stability of the solutions being studied. Solutions of blood in distilled water may give a desirable pH value, but the low electrolyte concentration so obtained would favor the precipitation of globulins and thus cause a turbid solution. Preliminary experiments indicated that solutions of oxyhemoglobin between pH values of 6 and 8 were stable. Since lower hydrogen-ion concentrations favor the formation of oxyhemoglobin, the pH level of 8.0 was chosen for further study.

In order to have an adequate electrolyte concentration and, in addition, to have a known hydrogen-ion concentration, it was felt that a dilute buffer solution was indicated. Such a buffer must, however, be dilute enough to permit the laking of red cells in a reasonable time.

The buffer solution we have found most suitable is a mixture of 947 c.c. of M/15 Na_2HPO_4 and 53 c.c. M/15 KH_2PO_4.[6] This buffer can be used in two ways. We have for the past year laked 0.1 c.c. blood in 15 c.c. distilled water, and after hemolysis is complete (2 to 3 minutes at 25 to 30° C.) 5 c.c. of the buffer are added. Since this involves two solutions we attempted to find the minimal concentration of buffer that might be incorporated in the laking fluid. It was found that a 1:20 dilution of the stock buffer permitted the use of 20 c.c. of this solution as the laking fluid.

The recent commercial production of tablets for the preparation of buffers suggested the use of such material for the buffer solution. The Certified Buffer Tablets* for a pH of 8.0 are composed of sodium tetraborate and potassium acid phosphate. Instructions that accompany such tablets call for the dilution of the tablet in 100 c.c. of warm distilled water. Such a buffer solution does not permit cell hemolysis. However, if a 1:20 dilution of the concentrated buffer solution is made, 20 c.c. of this dilute buffer may be used as the hemoglobin solvent.

In Table II is shown the relative effectiveness of carbonate, diluted M/15 buffer, and the diluted commercial tablets in maintaining the constancy of oxyhemoglobin when the samples were kept at 40° C. for the indicated times. This

TABLE II. HEMOGLOBIN VALUES OBTAINED BY DILUTING 0.1 C.C. SAMPLES OF A BLOOD SPECIMEN WITH THREE SOLVENTS: (1) 0.1 PER CENT SODIUM CARBONATE, (2) DILUTED M/15 PHOSPHATE BUFFER, AND (3) BUFFER TABLET IN 2,000 C.C. H_2O; SAMPLES KEPT AT 40° C. FOR THE INDICATED PERIODS OF TIME

TIME (MIN.)	0.1 PER CENT CARBONATE	DILUTED PHOSPHATE BUFFER	DILUTED BUFFER TABLET
0	12.9	12.9	12.9
15	12.6	12.9	12.9
30	12.5	12.9	12.9
45	12.1	12.9	12.9
60	11.8	12.9	12.9
75	11.7	12.9	12.9
90	11.5	12.9	12.9
105	11.3	12.9	12.9
120	11.1	12.9	12.9
270	9.8	12.9	12.9

*Supplied through the courtesy of the Coleman Electric Company, Maywood, Ill.

is merely a group of representative data which are comparable to many similar experiments.

Similar experiments where 0.1 c.c. blood was laked with 15 c.c. water, 5 c.c. of the M/15 buffer subsequently being added, gave results comparable to the dilute phosphate and tablet buffer solutions listed in Table II.

CONCLUSIONS

1. Blood diluted 1:200 in 0.1 per cent carbonate, having a pH of 10.5, is unstable at elevated temperatures. Data are presented which indicate that the oxyhemoglobin is probably converted first to methemoglobin and later to hematin derivative.

2. If the pH of the solvent is lowered to approximately 8.0, the oxyhemoglobin solutions are stable over a prolonged period.

Appreciation is expressed to Miss Emily Flanagan for her help in this work.

REFERENCES

1. Heilmeyer, Ludwig: Spectrophotometry in Medicine. Translated by Jordan, A., and Tippell, T. L., London, Eng., Adam Hilger, Ltd. Supplied by Jarrell-Ash Co., Boston, Mass.
2. Sheard, C., and Sanford, A. H.: A Photo-Electric Hemoglobinometer Clinical Application of the Principles of Photo-Electric Photometry to the Measurement of Hemoglobin, J. Lab. & Clin. Med. **14**: 558, 1929.
3. Ray, G. B., Blair, H. A., and Thomas, C. I.: The Spectrophotometric Determination of Certain Blood Pigments, J. Biol. Chem. **98**: 63, 1932.
4. Drabkin, D. L., and Austin, J. Harold: Spectrophotometric Studies. I. Spectrophotometric Constants for Common Hemoglobin Derivatives in Human, Dog, and Rabbit Blood, J. Biol. Chem. **98**: 719, 1932.
5. Martini, P., and Schuler, B.: Spektralphotometrische Blutuntersuchungen. Die Veränderung des Oxyhamoglobins unter dem Einfluss ultra-violetter Strahlen, Ztschr. f. d. ges. exper. Med. **83**: 211, 1932.
6. Hastings, A. B., and Sendroy, J.: Studies of Acidosis. XX. The Colorimetric Determination of Blood pH at Body Temperature Without Buffer Standards, J. Biol. Chem. **61**: 695, 1924.

THE DETERMINATION OF THE FREE AND TOTAL CHOLESTEROL OF PLASMA WITH THE PHOTOELECTRIC COLORIMETER

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IN UNDERTAKING a prolonged and intensive study of chronic liver disease, it was desired to include among our tests of liver function the estimation of the free and total cholesterol of the plasma. Sperry[1, 2] has demonstrated that, when a reliable method of determination is employed, the percentage or ratio of free to total cholesterol is maintained at an almost constant value in the normal adult serum or plasma and that relatively slight variations from this value are indicative of some abnormality in cholesterol metabolism. Since the magnitude of the variation which is significant may therefore be small, it is essential to employ a technique which invariably yields uniform results. The method presented here has proved satisfactory and reliable in our hands, and with the present wide-

From the Department of Medicine, New York University College of Medicine.
The research was aided by grants from the Milbank Memorial Foundation and the Nutrition Foundation.
Received for publication March 17, 1945.

spread use of the photoelectric colorimeter, it was thought that other laboratories might find it equally useful.

In 1934, Schoenheimer and Sperry[3] published a method for the determination of free and combined cholesterol which was based on an initial precipitation as the digitonide followed by the application of the familiar Liebermann-Burchard reaction to its colorimetric estimation. Their procedure gave more uniform results than had heretofore been obtained. The method presented by us is essentially a modification of that of Schoenheimer and Sperry. Certain features which have been incorporated were suggested by Sperry in a later publication[4] and by Alber and Bryant.[5] Data obtained from thirty-five normal adults agree well with those obtained by Sperry.[1] In addition, a prolonged study of chronic liver disease has enabled us to gain evidence for the constancy of results obtainable by this method when applied repeatedly to the same individual over a long period of time.

METHOD

The method is described for the simultaneous determination of the cholesterol partition in two or more samples of plasma.

Reagents.—

1. Acetone-absolute alcohol (1:1)
2. Acetone-ether (1:2)
3. Ethyl ether (reagent grade)
4. Glacial acetic acid
5. 0.2 per cent digitonin in 50 per cent ethyl alcohol*
6. 100 per cent KOH in distilled water
7. 1 per cent phenolphthalein in 95 per cent ethyl alcohol
8. 5 per cent HCl solution
9. Acetic anhydride (reagent grade†)
10. Concentrated H_2SO_4 (reagent grade)

Procedure.—

Extraction of Plasma or Serum: For each 2 c.c. of plasma or serum, about 40 c.c. of acetone-alcohol are placed in a 50 c.c. volumetric flask and the plasma or serum pipetted into this, drop by drop, with constant shaking. Additional acetone-alcohol is added to just below the calibration mark on each flask and extraction allowed to take place overnight at room temperature. These unfiltered extracts are quite stable and may be kept for some days if necessary. After extraction, the flasks are made up to volume and the contents well mixed and filtered through lipid-free filter paper. Evaporation is avoided by keeping the funnels covered and working rapidly.

Precipitation of Free Cholesterol: Ten cubic centimeters of each filtrate are pipetted into 30 c.c. conical centrifuge tubes, 4 c.c. of the digitonin solution added to each, and the mixture stirred well. The rods are left in the tubes which are placed in one or more covered Mason jars, containing a layer of sand, and left overnight at room temperature. The rods are then removed to a numbered rack or nail after both rods and walls of the tubes have been washed down with acetone to free the precipitates. Care must be taken to return each rod to the correct tube in order to avoid the loss of any precipitate which may have adhered to it. The tubes are then centrifuged for twenty minutes at high speed, after

*Hoffman-La Roche, Inc., Nutley, N. J., or Merck & Co., Inc., Rahway, N. J.
†Eastman Kodak Co., Rochester, N. Y.

which the supernatant fluid is removed with a fine capillary pipette and suction. The stirring rods are replaced in the tubes; rods and tube walls are washed down with 2 to 4 c.c. of acetone-ether. After thorough stirring, the rods are removed and washed down with a small amount of acetone-ether. The tubes are centrifuged at high speed for ten minutes, the supernatant fluid removed as before, and this washing repeated two more times, each time with plain ether. After the final washing, the tubes are placed in a water bath at 40° C. for a few minutes to dry, after which the rods are replaced. Tubes can be left at this stage for some days if kept covered in Mason jars.

Precipitation of Total Cholesterol: Five cubic centimeter aliquots of the original filtrates are pipetted into 30 c.c. conical centrifuge tubes which are either graduated or have been marked to contain 9 c.c. A fresh mixture of one part 100 per cent aqueous KOH and three parts absolute alcohol is made up and to each tube are added fifteen drops of this alcoholic KOH. The tubes are well stirred and placed in Mason jars which contain a layer of sand and which have been heated previously to 40° C. The jars are covered tightly and placed in an incubator at 37 to 40° C. for from three to four hours for saponification. Following this, the tubes are cooled and acetone-alcohol is added to the 9 c.c. mark. This is best done with a dropper, washing down the sides of the tubes. One drop of phenolphthalein solution is added to each tube and titrations carried out with 5 per cent HCl. To insure a slight excess of acid, one to two drops are added after the color change. Four cubic centimeters of digitonin solution are added to each, the mixtures well stirred, and the tubes placed in covered jars and allowed to stand overnight at room temperature. From this point, centrifugation and washing are carried out as for free cholesterol.

Precipitation of Standard Cholesterol: In our experience, it has been found best to prepare a standard cholesterol curve as follows: Standard cholesterol solutions containing 0.2, 0.4, and 0.6 mg. of cholesterol in 10 c.c. of acetone-alcohol are carried through the determination exactly as for free cholesterol. Initially, it was thought wise to do this with each set of determinations, but as we gained experience with the method, this was found to be unnecessary. It is certainly to be recommended whenever new reagents are prepared or when other possible variables are introduced.

Development and Reading of Color: The tubes are removed from the jars and the jars then placed in a constant temperature oven and heated to from 100 to 110° C. The tubes are replaced in the hot jars and left in the oven for one-half hour. The jars are removed from the oven at the end of this time and the precipitates dissolved in glacial acetic acid as follows: 2 c.c. of the acid are added to each, being careful to wash down the walls of the tubes and the stirring rods. The precipitates are stirred well into the solvent and the tubes replaced in the hot sand in the jars for about one minute. The tubes are then placed in a water bath at about 60° C. until complete solution is effected. After solution of all of the precipitates, the tubes are transferred to a water bath at 25° C. While temperature equilibration is being attained, a fresh mixture of acetic anhydride-sulfuric acid is made up in proportions of 40 volumes of acetic anhydride to 1 volume of concentrated sulfuric acid. A sufficient quantity is prepared to permit the addition of 8 c.c. to each tube. This reagent is stable for no longer than one hour at room temperature. To the first tube are added 8 c.c. of the acetic anhydride-sulfuric acid mixture, the contents well stirred, and the tube immediately placed in a water bath at 25° C. in the dark. The remaining tubes are treated similarly, the time of the addition of reagent to the first tube

and the approximate interval between tubes being noted. A reagent blank of 2 c.c. of glacial acetic acid and 8 c.c. of acetic anhydride-sulfuric acid is prepared at the same time. At approximately twenty-five minutes after the addition of reagent to the first tube, the contents of the tubes are transferred to photoelectric colorimeter tubes, protecting the solutions at all times from exposure to light. Colorimeter readings are made on the tubes in the same sequence in which the final reagent was added and are begun thirty minutes after the addition of this reagent to the first tube. After each has been read, readings are repeated in the same sequence until maximal color intensity has been obtained and the reading is stable. We employ an Evelyn photoelectric colorimeter with a light filter having maximal transmission at 660 mu.

Calculation of Results: A standard curve is prepared, as noted, from readings obtained on known cholesterol solutions run through the method as for free cholesterol. Color intensity (in units appropriate to the type of colorimeter employed) is plotted against milligrams of cholesterol. That portion of the curve corresponding to a concentration of 0.2 to 0.6 mg. of cholesterol yields satisfactory results with the Evelyn colorimeter and the 660 light filter. The amount of cholesterol present in the aliquot of filtrate used is then read off directly from this curve.

RESULTS

Accuracy.—To determine the completeness of recovery from pure cholesterol solutions by this method, standard solutions of cholesterol in acetone-alcohol were treated as for free cholesterol and the resultant readings referred to a curve obtained simultaneously from solutions of pure cholesterol dissolved in glacial acetic acid. In Table I are given the results of an experiment of this type.

To check the accuracy of recovery of cholesterol added to plasma, a known quantity of cholesterol in acetone-alcohol was transferred to a 50 c.c. volumetric flask, additional acetone-alcohol added, and 2 c.c. of plasma pipetted into this solution. A separate aliquot of the same plasma was extracted without added cholesterol. The two extracts were carried through the method as described. In the experiments as performed, 1 mg. of cholesterol was added to the extraction flasks, which is equivalent to increasing the plasma values by 50 mg. per cent. Results so obtained with three different plasmas are given in Table II.

TABLE I

CHOLESTEROL DISSOLVED IN ACETONE-ALCOHOL (MG.)	CHOLESTEROL RECOVERED (MG.)	PER CENT RECOVERY
0.200	0.198	99.0
0.300	0.291	97.0
0.400	0.385	96.0
0.500	0.483	98.6
0.600	0.596	99.3

TABLE II

	PLASMA CHOLESTEROL (MG. %)	PLASMA PLUS 50 MG. % CHOLESTEROL CALCULATED (MG. %)	DETERMINED (MG. %)	PER CENT RECOVERY
A.	Free 67	117	117	100.4
	Total 225	275	276	100.4
B.	Free 67	117	115	98.3
	Total 219	269	271	100.7
C.	Free 34	84	85	101.2
	Total 126	175.5	173	98.6

Plasma Cholesterol Partitions of Normal Adults.—In Table III are recorded the values for the free, total, and free in total cholesterols obtained by the application of this method to the plasmas of thirty-five normal adult human beings of both sexes, ranging in age from 18 to 63 years. These values agree quite well with those reported by Sperry,[1] who reports a mean value for free in total cholesterol of 26.9 per cent with a standard deviation of ± 1.4.

TABLE III. PLASMA CHOLESTEROL—NORMAL ADULTS

SUBJECT	FREE CHOLESTEROL (MG. %)	TOTAL CHOLESTEROL (MG. %)	FREE IN TOTAL CHOLESTEROL (%)
1	79	300	26
2	31	120	26
3	73	250	29
4	30	123	24
5	40	153	26
6	84	288	29
7	46	188	24
8	37	167	22
9	59	217	27
10	38	150	25
11	50	200	25
12	41	157	26
13	69	250	28
14	43	192	22
15	54	200	27
16	45	185	24
17	68	263	26
18	51	183	28
19	31	137	23
20	53	197	27
21	46	170	27
22	49	173	28
23	45	171	26
24	56	217	26
25	36	167	22
26	71	262	27
27	45	175	26
28	66	230	29
29	46	180	26
30	56	198	28
31	37	145	26
32	38	161	24
33	77	262	29
34	61	218	28
35	50	189	26

Mean, 26.1.
Standard deviation, ± 1.9.
Coefficient of variation, 7.3 per cent.

Constancy of the Partition.—Finally, in Charts I and II evidence is presented for the constancy with which a given individual maintains the percentage of free in total cholesterol over an extended period of time regardless of the total cholesterol values. The subjects used for this experiment were patients with cirrhosis of the liver on the wards of the Third (New York University) Medical Division of Bellevue Hospital. Chart I is compiled from figures obtained from a patient who, over a period of twenty-six months, has shown some clinical response to the treatment of cirrhosis, but none of whose laboratory data gives evidence of significant improvement in liver function. This patient might be said to be maintained in an approximately stationary condition, and it should be noted that all of the values for free in total cholesterol are abnormal. Chart II represents data from a patient who responded well to the treatment of cirrhosis both clinically and as judged by laboratory data. In this case the figures obtained after the second month of observation show a normal percentage of free in total cholesterol.

The results show clearly that although there may be considerable variation in total cholesterol, the percentage of free in total can be maintained within a remarkably narrow range, the level of which appears to correlate well with the clinical status of the patient suffering from cirrhosis of the liver.

CHART I - P.G.

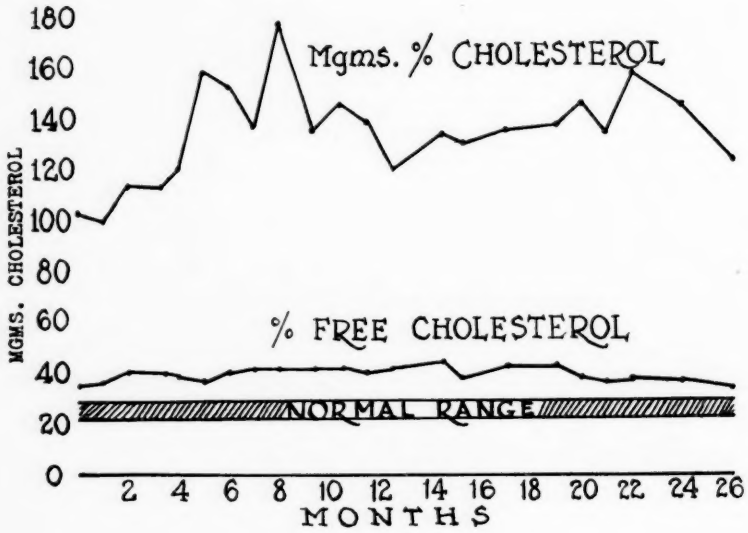
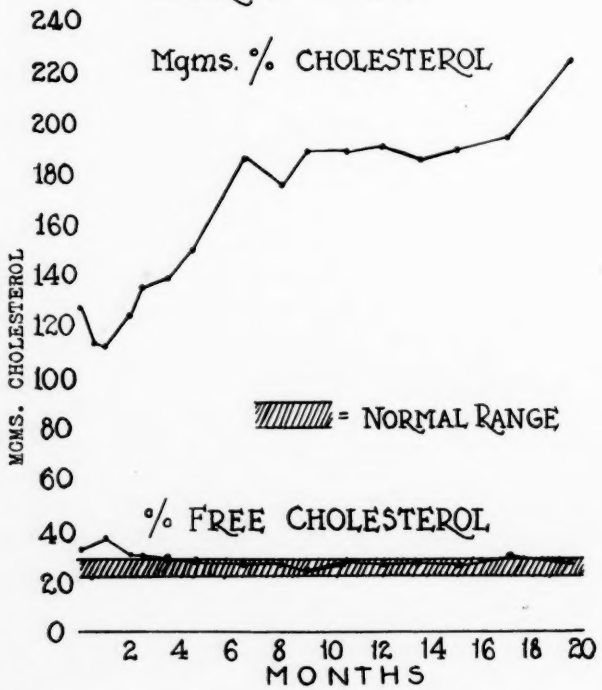


CHART II - W.W.



DISCUSSION

Since this method is presented as a modification of that of Schoenheimer and Sperry, discussion will, in the main, be limited to a consideration of the differences between our technique and the original.

Reagents.—The use of a 0.2 per cent digitonin solution in 50 per cent ethyl alcohol, as suggested by Alber and Bryant,[5] has proved satisfactory and is much simpler to prepare than the aqueous reagent of Schoenheimer and Sperry, since the digitonin dissolves fairly readily in the alcohol medium.

Shapiro, Lerner, and Posen[6] proposed the use of a fresh mixture of acetic anhydride and sulfuric acid instead of the separate addition of the two reagents. This departure leads to greater uniformity of results since it avoids the undesirable evolution of heat in the presence of cholesterol, where temperature control is important and also eliminates the difficulties inherent in the accurate measurement of a small volume of the acid. Sperry and Brand[7] has apparently come to the same conclusion.

Amounts Employed.—The volumes of plasma, extracts, and reagents given in our method are dependent upon the sensitivity of the photoelectric colorimeter employed and upon the final volume necessary with this colorimeter. These can undoubtedly be modified according to the instrument to be used.

Extraction of Plasma.—We have found it unnecessary to use heat in the extraction of plasma if the plasma-acetone-alcohol mixture is allowed to stand overnight at room temperature.

Total Cholesterol.—The time periods for saponification and for precipitation of total cholesterol are considerably longer than those employed by Schoenheimer and Sperry. We have not investigated the possibility of shortening either, since these fit in well with the schedule for the entire determination and since completion of both processes is thus ensured.

Reading of Color.—It should be noted that, according to our method, repeated readings of an entire series are taken until the readings become maximal and stable. We have been unable to find a single time interval which will give uniform results. One explanation for this is that the time required for maximal color development, under our conditions, varies with the amount of cholesterol present. Also, since it is well established that temperature has a marked effect upon the rate of color development, it is probable that slight uncontrolled day-to-day variations in this factor have influenced our results despite efforts at control. However, repeated readings, even of a large series, are easily taken and the results so obtained with standard solutions yield values of a gratifying constancy, so that we feel this to be a satisfactory point of departure.

Standard Curve.—Our standard curve is prepared from cholesterol precipitated as the digitonide prior to color development. In our experience, the values obtained from such a curve are subject to much less variation than those obtained from free cholesterol dissolved directly in glacial acetic acid. In fact, after familiarity with the method is gained, it is found that the digitonide curve is capable of quite accurate day-to-day reduplication.

SUMMARY

1. The details are given of a modification of the Schoenheimer-Sperry method for the determination of free and total cholesterol in plasma or serum.

2. The results of the analyses of thirty-five normal human plasmas by this method are presented.

3. The constancy at which an individual can maintain the percentage of free to total cholesterol in his plasma is demonstrated by two extended studies on patients with cirrhosis of the liver.

4. The major points of departure of this method from the original are discussed.

We wish to express our indebtedness to Dr. Charles Hoagland, of the Rockefeller Institute for Medical Research, for the use of some of the normal figures obtained by him with our method.

REFERENCES

1. Sperry, W. M.: The Relationship Between Total and Free Cholesterol in Human Blood Serum, J. Biol. Chem. **114**: 125, 1936.
2. Sperry, W. M.: The Effect of Dextrose Ingestion on the Cholesterol Fractions of the Blood, J. Biol. Chem. **116**: 65, 1936.
3. Schoenheimer, R., and Sperry, W. M.: A Micromethod for the Determination of Free and Combined Cholesterol, J. Biol. Chem. **106**: 745, 1934.
4. Sperry, W. M.: A Micromethod for the Determination of Total and Free Cholesterol, Am. J. Clin. Path. **8**: 91, 1938.
5. Alber, H. K., and Bryant, J. T.: The Micro Determination of Cholesterol in Serum, J. Franklin Inst. **229**: 118, 1940.
6. Shapiro, A., Lerner, H., and Posen, E.: A Fixed Color Standard for Cholesterol Determination, Proc. Soc. Exper. Biol. & Med. **32**: 1300, 1935.
7. Sperry, W. M., and Brand, F. C.: The Colorimetric Determination of Cholesterol, J. Biol. Chem. **150**: 315, 1934.

SIMPLIFICATION OF U. S. P. XII MICROBIOLOGICAL VITAMIN ASSAYS

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THE methods for the microbiologic assays of nicotinic acid and riboflavin, outlined in the first bound supplement of the U. S. P. XII, follow procedures widely disseminated in the literature.

Much of the basic work was done by Snell and Strong[1] in assaying riboflavin, Snell and Wright[2] in determining nicotinic acid, and Landy and Dicken[3] in devising a method for the assay of six B vitamins.

The above procedures, as well as those of the U. S. P. XII,[4] are based on the production, by the test microorganism, of acids in series of tubes of basal media containing varying amounts of unknown samples. Acidities are determined by titration with 0.1 N alkali, and potencies are derived by plotting against a standard curve constructed from acidities obtained from dilutions of the standard vitamin in the basal medium. The detailed methods of assay for nicotinic acid and riboflavin, used throughout the following experiments, are very competently described in the first bound supplement of the U. S. P. XII. The only deviation in our procedure in the nicotinic acid assay was to employ thirteen tubes in each standard series and eight tubes in each unknown series as contrasted to the U. S. P. XII requirement of eleven and four in the respective series of known and unknown.

Titrations may be made colorimetrically with bromthymol blue as the indicator or electrometrically with a glass electrode. The latter method, using a Beckman Laboratory Model G potentiometer with a system outlined by McQuarrie and Konen,[5] in which glass electrodes and burettes are in an ingenious arrangement, is to be preferred and is used routinely in this laboratory.

From The Wyeth Institute of Applied Biochemistry,
Received for publication, Feb. 5, 1945.

Pederson and Bagg[6] point out that many factors affect the relationship of titratable acidity and the final hydrogen-ion concentration in the growth of lactic acid bacteria. The buffer content of the medium and the proportional amounts of acids, especially lactic and acetic acids, that are produced during growth are most important. The varying amounts of lactic and acetic acids elaborated are especially dependent on the type of sugar incorporated in the medium and on the particular species of lactic acid bacteria used as the test organism. It is also noteworthy that when lactic acid is the primary acid a higher hydrogen-ion concentration results than when acetic acid is produced in quantity.

The observations of Pederson and Bagg are most important in the selection of a medium and of a test organism but, once that is accomplished, the necessity for titration in the vitamin assays is superfluous. Since the control standard is identical to the unknown sample, with the exception of the concentration of the vitamin being tested, the relationship between titratable acidity and hydrogen-ion concentration, under the conditions of test, should be constant. Consequently, substitution of a curve of pH values for titration values should suffice.

To verify this contention, data from sixty-eight nicotinic acid and twenty-four riboflavin determinations have been accumulated in which all samples were set up as in the U. S. P. XII methods, and potency determinations were made by plotting both pH and titration values.

The samples assayed included C. T. nicotinamide, C. T. nicotinic acid, solutions of nicotinic acid and riboflavin, yeast extracts, Plebex elixir, and Plebex tablets.

Nicotinic Acid.—The data in Table I are examples of results that have been obtained in the assay of nicotinic acid.

TABLE I

NICOTINIC ACID SAMPLE	NUMBER	(A) U. S. P. XII ACID TITRATION METHOD	(B) pH METHOD	PER CENT DEVIATION OF (B) FROM (A)
C. T. nicotinamide	435304	57.8 mg.	57.1 mg.	−1.2
C. T. nicotinic acid	441324	27.75 mg.	26.5 mg.	−4.4
Nicotinic acid (ampule)	1340237	2,140 mg.	2,160 mg.	+0.9
Yeast extract dried	91313	3,920 mg.	3,920 mg.	0
Plebex elixir	440039	435 mg.	430 mg.	−1.0
Plebex tablets	441857	1,040 mg.	1,040 mg.	0
Of 68 nicotinic assays				
39 assays average				−2.8
29 assays average				+2.0

TABLE II. TITRATION AND pH VALUES FOR NICOTINIC ACID STANDARD

TUBE	NICOTINIC ACID	pH*		ML. 0.1 N ACID†	
	μG	A	B	A	B
1	0.00	5.59	5.60	0.91	1.07
2	0.05	5.12	5.13	2.44	2.34
3	0.10	4.89	4.85	3.56	3.66
4	0.15	4.71	4.71	4.78	4.78
5	0.20	4.59	4.56	5.74	5.69
6	0.25	4.51	4.45	6.15	6.51
7	0.30	4.43	4.43	6.97	6.97
8	0.35	4.39	4.35	7.47	7.42
9	0.40	4.31	4.32	8.09	7.68
10	0.45	4.29	4.26	8.39	8.44
11	0.50	4.28	4.23	8.85	8.80
12	0.00‡	6.66	6.66	–	–
13	0.00‡	6.66	6.67	–	–

*See Fig. 1.
†See Fig. 2.
‡Uninoculated blanks.

In Table II are included the pH values and acid volumes produced by standard nicotinic acid. These data are plotted in the corresponding Figs. 1 and 2. To facilitate accurate plotting and reading, the curves were drawn on Keuffel and Esser graph paper, No. 358-14. Figs. 1 and 2 were employed in the computation in Table III of the nicotinic acid content of Plebex tablets No. 441857, which were coated tablets containing Anheuser-Busch yeast extract powder with added thiamine HCl and riboflavin.

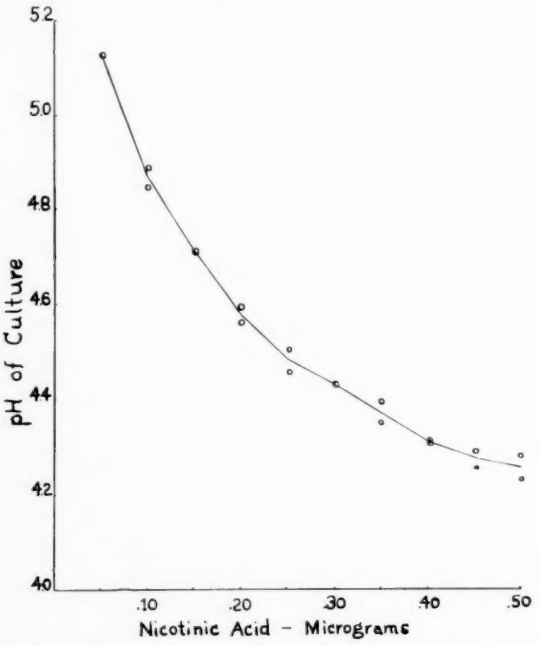

Fig. 1.—Standard nicotinic acid curve plotting pH values of cultures.

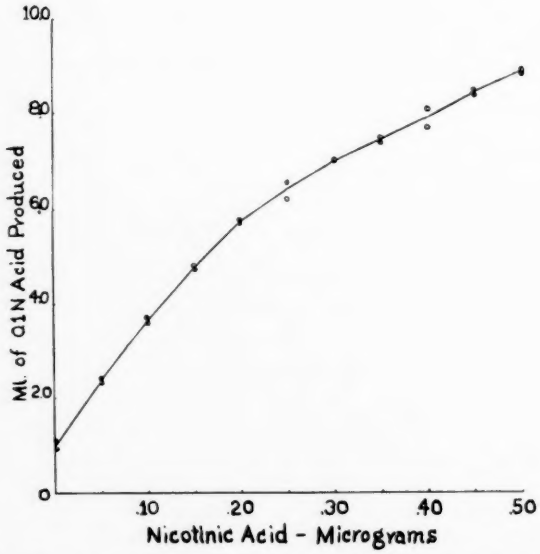

Fig. 2.—Standard nicotine acid curve plotting titratable acid of cultures.

Thirteen of the sixty-eight nicotinic acid assays were also checked by a turbidimetric method. Additional series were set up following U. S. P. XII procedure but, instead of titrating, the turbidity of each was determined with a

TABLE III. COMPUTATION OF NICOTINIC ACID CONTENT OF PLEBEX TABLET NO. 441857 BY pH AND ACID TITRATION METHODS

TUBE	ML. DILUTED SAMPLE*	pH		μG. PER CULTURE†		μG PER ML. DILUTED SAMPLE		ML. 0.1 N ACID		μG PER CULTURE‡		μG PER ML. DILUTED SAMPLE	
		A	B	A	B	A	B	A	B	A	B	A	B
1	1.0	4.89	4.88	.096	.098	.096	.098	3.56	3.66	.098	.102	.098	.102
2	1.5	4.71	4.69	.150	.157	.100	.105	4.63	4.83	.143	.153	.095	.102
3	2.0	4.52	4.52	.228	.228	.114	.114	5.14	5.34	.170	.182	—	—
4	2.5	4.48	4.45	.249	.278	.100	.111	6.36	6.66	.253	.277	.100	.111
5	3.0	4.41	4.41	.307	.307	.102	.102	7.17	7.17	.322	.322	.107	.107
6	3.5	4.36	4.35	.360	.377	.103	.108	7.63	7.63	.370	.370	.106	.106
7	4.0	4.31	4.32	.400	.400	.100	.100	7.78	7.73	.386	.382	.097	.096
8	5.0	4.24	—§	—	—	—	—	—	—	—	—	—	—
					Mx	.102	.106				Mx	.100	.107
					Mx A & B		.104				Mx A & B		.104
.104 × 10,000 (dilution factor) = 1,040 μg										.104 × 10,000 (dilution factor) = 1,040 μg			

*Dilution of Sample 1-10,000.
†Data from Fig. 1.
‡Data from Fig. 2.
§Off curve.

Klett-Summerson colorimeter. Seven assays showed an average deviation of –3.4 per cent from the U. S. P. XII assay, while six showed an average deviation of +3.4 per cent. Turbidimetric assay data have not been included because of the obvious advantages of the pH method.

Riboflavin.—Of twenty-four assays for riboflavin, determined by both U. S. P. XII and pH methods, six pH assays averaged a –0.6 per cent deviation

TABLE IV. TITRATION AND pH VALUES FOR RIBOFLAVIN STANDARD

TUBE	RIBOFLAVIN (μG)	pH*		ML. 0.1 N ACID†	
		A	B	A	B
1	0.00	5.96	6.03	0.82	0.40
2	0.05	5.13	5.13	2.28	2.34
3	0.10	4.82	4.82	3.87	3.92
4	0.15	4.59	4.56	5.39	5.54
5	0.20	4.40	4.38	6.71	7.07
6	0.25	4.20	4.19	8.69	8.69
7	0.30	4.12	4.12	9.25	9.30
8	0.50	4.03	4.03	–	–
9	0.00‡	6.62	6.63	–	–
10	0.00‡	6.63	6.67	–	–

*See Fig. 3.
†See Fig. 4.
‡Uninoculated blanks.

TABLE V. COMPUTATION OF RIBOFLAVIN CONTENT OF PLEBEX TABLET NO. 441857 BY pH AND ACID TITRATION METHODS

TUBE	ML. DILUTED SAMPLE*	pH		μG. PER CULTURE†		μG PER ML. DILUTED SAMPLE		ML. 0.1 N ACID		μG PER CULTURE‡		μG PER ML. DILUTED SAMPLE	
		A	B	A	B	A	B	A	B	A	B	A	B
1	0.5	5.11	5.10	.053	.054	.106	.108	2.39	2.44	.052	.053	.104	.106
2	1.0	4.80	4.80	.104	.104	.104	.104	3.87	3.92	.099	.100	.099	.100
3	1.5	4.51	4.61	.167	.143	.101	.095	5.79	5.04	.162	.137	.108	.091
4	2.0	4.32	4.40	.218	.196	.109	.098	7.43	6.82	.215	.197	.108	.099
5	2.5	4.21	4.17	.246	.260	—	—	8.44	9.15	.244	—	—	—
6	3.0	—§	—	—	—	—	—	—	—	—	—	—	—
7	3.5	—	—	—	—	—	—	—	—	—	—	—	—
8	4.0	—	—	—	—	—	—	—	—	—	—	—	—
					Mx	.105	.101				Mx	.105	.099
					Mx A & B		.103				Mx A & B		.102
.103 × 1500 (dilution factor) = 155 μg										.102 × 1500 (dilution factor) = 153 μg			

*Dilution of Sample 1-1500.
†Data from Fig. 3.
‡Data from Fig. 4.
§Off curve.

from the U. S. P. XII while eighteen pH assays averaged a ±3.5 per cent deviation from the U. S. P. XII results.

In Table IV are listed the pH values and acid volumes produced by standard riboflavin. The corresponding Figs. 3 and 4 were used in the computation in Table V of the riboflavin content of Plebex tablets No. 441857.

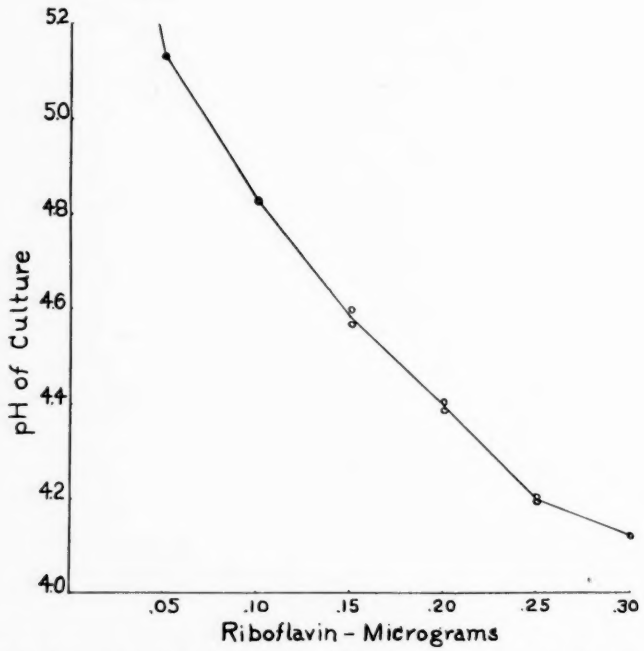

Fig. 3.—Standard riboflavin curve plotting pH values of cultures.

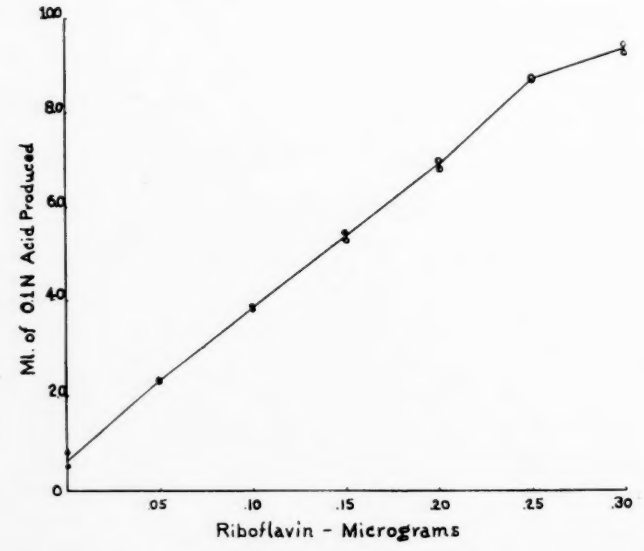

Fig. 4.—Standard riboflavin curve plotting titratable acid of cultures.

As with the nicotinic acid determinations, a small number of U. S. P. XII assays were checked very closely by turbidimetric methods.

Since the actual setup and calculations for all three methods are the

same, any saving in time occurs in the reading of the results. The following comparison shows the superiority of the pH method:

METHOD	TIME REQUIRED FOR READING
U. S. P. XII	45 to 55 minutes for the duplicate standard 30 to 35 minutes for each duplicate unknown
Turbidimetric	25 to 30 minutes for the duplicate standard 15 minutes for each duplicate unknown
pH	15 to 20 minutes for duplicate standard 10 minutes for each duplicate unknown

An obviously additional advantage to the pH method, of course, lies in a minimum of manipulation with, consequently, smaller expected error.

CONCLUSIONS

1. It is possible to obtain comparable results in the U. S. P. XII microbiologic vitamin assays by the substitution of pH values for titration values in the construction of the curve for potency determination.

2. Such a procedure has the advantage of requiring less time and manipulation in microbiologic vitamin assays.

REFERENCES

1. Snell, E. E., and Strong, F. M.: A Microbiological Assay for Riboflavin, Indust. & Engin. Chem. (Anal. Ed.) **11**: 346, 1939.
2. Snell, E. E., and Wright, L. D.: A Microbiological Method for the Determination of Nicotinic Acid, J. Biol. Chem. **139**: 675, 1941.
3. Landy, M., and Dicken, D. M.: A Microbiological Assay Method for Six B Vitamins Using Lactobacillus Casei and a Medium of Essentially Known Composition, J. Lab. & Clin. Med. **27**: 1086, 1942.
4. The First Bound Supplement of the U. S. P. XII, 75-81, 1943.
5. McQuarrie, E. B., and Konen, H. J.: Glass Electrode Assembly for Titrating Microbiological Vitamin Assays, Indust. & Engin. Chem. (Anal. Ed.) **16**: 205, 1944.
6. Pederson, C. S., and Bagg, J. V.: The Cause of Variation in the Relationship Between Titratable Acidity and Hydrogen Ion Concentration Among Lactic Acid Bacteria, J. Bact. **48**: 559, 1944.

AN OPTICAL FLUORESCENCE COMPARATOR

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THE use of atabrine as the principal drug in the treatment of malaria made it highly desirable that simple methods be found to determine the blood level of this drug in large series of patients. The efficiency of the treatment depends essentially on the quick establishment of a satisfactory blood level, and the control of suppressive treatment is only possible if blood levels can be taken at random in forward field hospitals.

The rapid and simple method for the differential diagnosis of bacterial meningitis, as it was recently described by one of us,[1] based upon the estimation of the fluorescein content of the spinal fluid, also requires a simple inexpensive instrument to approximate the fluorescence of the specimens. Many

Aided by a grant from the John and Mary R. Markle Foundation.

From the New York Medical College, Flower and Fifth Avenue Hospitals, and the Metropolitan Hospital (Research Unit).

Received for publication, March 31, 1945.

tests in clinical vitamin chemistry could be made available to smaller laboratories by the use of such an instrument. In addition, the examination of specimens for their atabrine content can hardly be carried out with a sensitive photoelectric instrument in forward field hospitals in tropical climates since these instruments are not able to stand extreme changes in temperature and humidity as well as violent fluctuations of line voltage. The same holds true for the Dermofluorometer,[2] which permits the estimation of atabrine levels in the skin, but which is also sensitive to extreme climatic changes. It seemed desirable, therefore, to design an instrument which would obviate the disadvantages of a photoelectric fluorescence meter without sacrificing too much accuracy.

The instrument described* permits the estimation of fluorescence by visually comparing the fluorescence of the unknowns with that of a series of fluorescent solutions of known concentration (standards). The optical fluorescence comparator then serves to indicate the place which the unknown takes in a series of these standards.

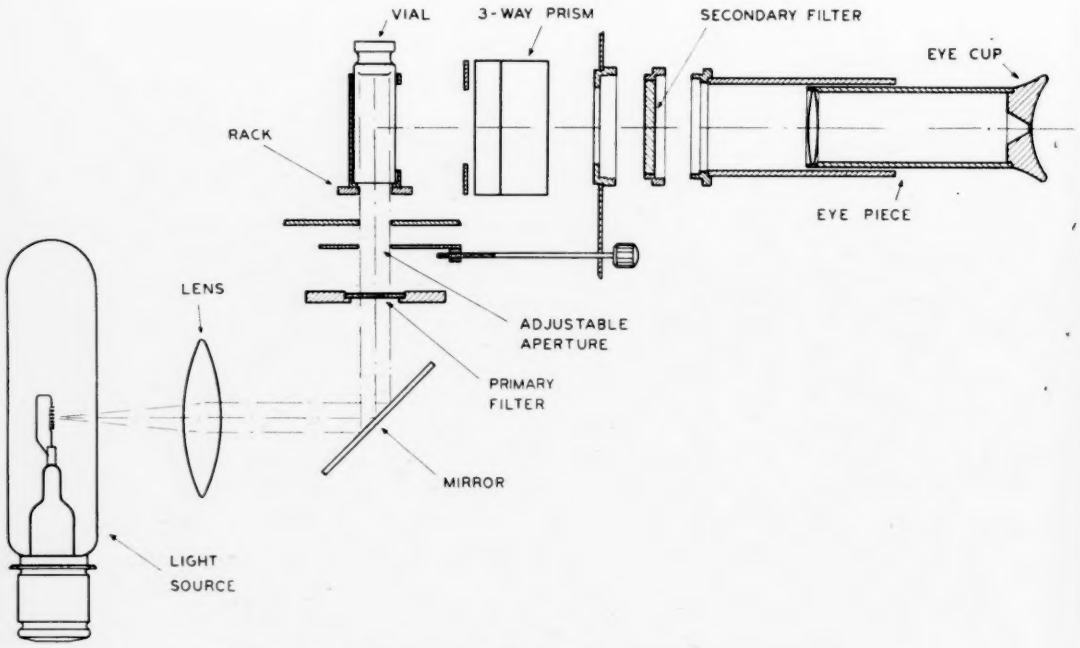

Fig. 1.—Schematic setup of the optical fluorescence comparator.

The light from an incandescent or mercury vapor lamp is collimated by a lens, is deflected by a mirror, and passes vertically through three flat-bottom vials of 5 c.c. content (Fig. 1). The fluorescence thus incited in the liquid is observed horizontally from the front. The optical design is such that the light beam is absolutely parallel and stays so when passing through the solutions in the vials which have ground bottoms. As a result of this, blanks appear perfectly black when viewed through the eyepiece.

A split-field optical-viewing system provides three fields of equal size, directly adjacent to each other (Fig. 2). The three vials brought together into the triple fields are viewed through an adjustable magnifying eyepiece. Primary and secondary filters can be introduced between light source and sample and sample and eyepiece, respectively. The device is constructed in a way that it

*Developed in cooperation with the Photovolt Corporation, New York, N. Y.

can be used with incandescent or a mercury vapor bulb, depending on what range of wave length of exciting light is desired. Using the incandescent bulb and a primary filter of Corning glass No. 5113 and a secondary filter of Corning glass No. 3480, 0.1 gamma per cent of atabrine extracted by the method of

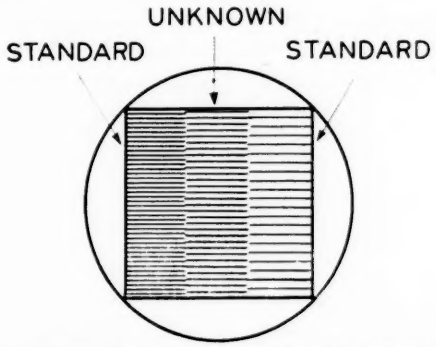

Fig. 2.—Appearance of the three samples through the eyepiece.

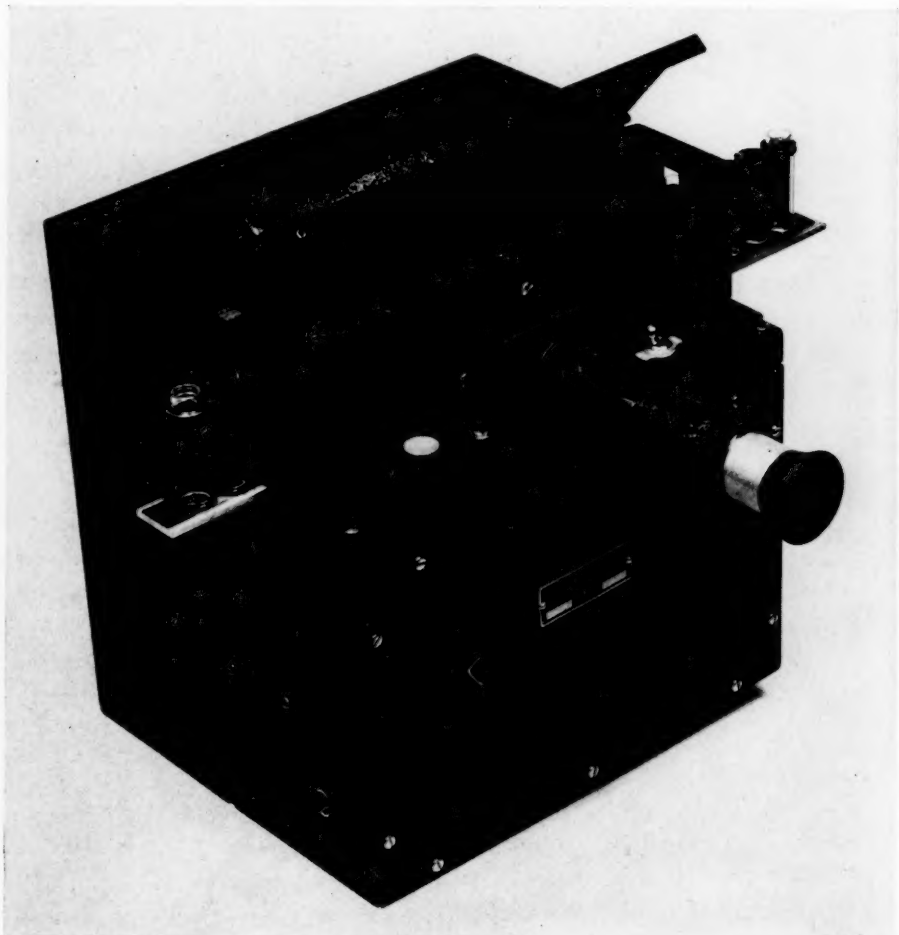

Fig. 3.—The optical fluorescence comparator.

Lange and Matzner can still be clearly recognized. It is possible that this sensitivity for atabrine can still further be improved by using substances that enhance the fluorescence, as described by Brodie and Udenfriend[3] and Auer-

bach and Eckert.[4] While the accuracy in determinations with a photoelectric fluorescence meter is obviously higher, the lowest detectable amount of a substance seems to be better with the fluorescence comparator, using the human eye as a registering element.

The operation of the instrument is simple and can be carried out by a person with little experience and irrespective of climate and variations in line voltage. The fluorescent standards of known concentration are poured into vials containing 5 c.c. each, and these are inserted in the order of increasing concentration into the sliding rack so that a space is left open between each two. The vial with the unknown is inserted into the space between two standards, and the three vials are then viewed, the two standards occupying the outside fields and the unknown the center field. If the brightness of the unknown is found not to be equal to one of the standards, or to be of intermediate fluorescence, the rack containing the standards is shifted and the unknown inserted into the next gap. This step is repeated until it is found that the unknown is either equal to one of the standards or is between two.

In order to lengthen the life of the exciting lamp, a powerful blower cools the bulb. Both are turned on only during the time of actual measurements by pressing a push button. Three adjustable apertures are provided under the vials to equalize the brightness of the field. This adjustment may only be necessary when the bulb is exchanged. Three vials are filled with the same fluorescent liquid and the knobs (Fig. 3) controlling the apertures are moved until the brightness is even. Once the three fields are equalized in this manner, readjustment will not be necessary, except after installing a new lamp.

SUMMARY

A visual fluorescence comparator is described which permits exact determinations of fluorescence of biologic materials as atabrine extracts or fluorescein plasma and spinal fluid samples. The device operates under all climatic conditions and is independent of fluctuations in line voltage.

REFERENCES

1. Lange, K., and Schwimmer, D.: A Simple Method for the Diagnosis of Acute Inflammatory Meningitis. In press.
2. Lange, K., and Krewer, S. E.: The Dermofluorometer, J. Lab. & Clin. Med. **28**: 1746-1750, 1943.
3. Brodie, B. B., and Udenfriend, F.: Estimation of Atabrine in Biological Fluids and Tissues, J. Biol. Chem. **151**: 299-317, 1943.
4. Auerbach, M. E., and Eckert, W. H.: The Photofluorometric Determination of Atabrine, J. Biol. Chem. **154**: 597-603, 1944.

A METHOD FOR DETERMINING THE COAGULATION TIME USING CAPILLARY BLOOD

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SEVERAL years ago one of us (T. K.) developed a method for determining the coagulation time using capillary blood. The current emphasis on changes in the coagulation time following the administration of heparin[1, 2] prompts us to publish the method at this time inasmuch as the frequent venepunctures necessary to follow the effect of heparin by the standard Lee-White method complicate the use of this anticoagulant. The inaccuracy of the commonly used capillary tube method[3, 4] because of the variable end point and inadequate temperature control is generally recognized.

METHOD

Thick-walled capillary tubes (40 by 5 mm.) with a 1 mm. bore and ground to a tapered point are fitted at one end with a short length of rubber tubing sealed with a glass plug (Fig. 1, *A*). After preliminary cleansing with alcohol,

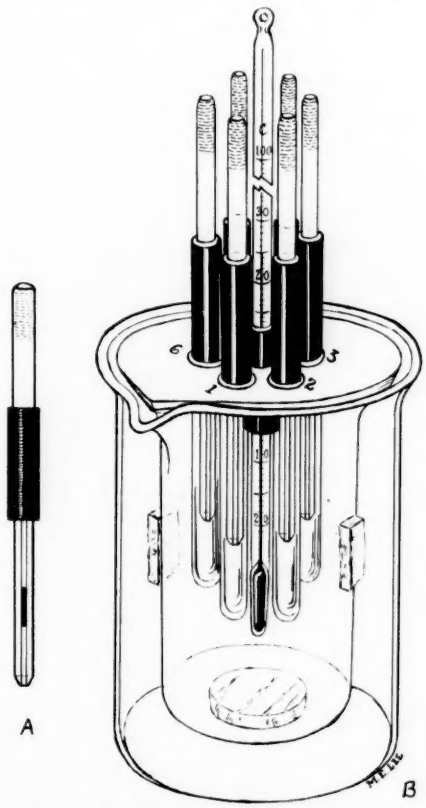

Fig. 1.—*A*, Capillary tube 40 by 5 mm. with a 1 mm. bore fitted with rubber tubing and glass plug as used in this method. *B*, Large empty beaker with an inner smaller beaker and fitted with a lid containing holes for six small, flared test tubes and a thermometer. Cork insulation is used to separate the inner and outer beakers. Cutler sedimentation tubes have proved satisfactory for the inner air jacket tubes.

From the Department of Physiology and Pharmacology of the School of Medicine of the University of Pittsburgh.

Received for publication, April 25, 1945.

the palmar surface of the distal phalanx is punctured, with the point of a No. 11 scalpel blade. This blade produces a free-flowing drop with a minimum of trauma and therefore little tissue fluid admixture. Squeezing the finger to obtain a drop of blood must be avoided because a decrease in the coagulation time from thirty seconds to one minute may be induced. A 1 cm. column of blood is drawn up to the midportion of the capillary tube by compressing and releasing the rubber tubing and partially removing the glass plug. Two samples are quickly taken from the same wound and placed immediately in a water bath at 25° C. At one-half minute intervals (or oftener as one approaches the coagulation time) the column of blood is displaced a short distance by compressing the rubber tubing. The end point is taken as that time when the column of blood cannot be displaced without leaving a definite stain on the glass wall. The average of the samples is taken as the coagulation time.

Two details must be observed if duplicate results are to be obtained: (1) the tubes must be uniformly clean and dry; (2) the temperature must be carefully controlled. To prevent the coagulated blood in the tubes from drying, immediately after using, they are placed in hydrogen peroxide in the vertical position. To clean the tubes thoroughly they are rinsed in forcefully running water, placed overnight in sulfuric acid-chromate cleaning solution, rinsed thoroughly, and air dried. If even soft wire is used to remove clot from the lumen of the tube it should be carefully done to avoid scratching the glass. To keep the temperature of the tubes at 25° C., a large empty beaker with an inner smaller beaker filled with water at 25° C. and fitted with a lid containing holes for six small test tubes and a thermometer has proved satisfactory (Fig. 1, *B*). Temperature control is necessary as a change from 20 to 28° C. may alter the coagulation time as much as 1.5 minutes. It is advisable to have the coagulation tubes stand in the water bath for from ten to fifteen minutes before using. The thick-walled glass permits the relatively small volume of blood to take on the temperature of the glass. A temperature of 25° C. has

TABLE I. SUCCESSIVE COAGULATION TIMES USING THE CAPILLARY TUBE METHOD DESCRIBED*

SUBJECT	TEST 1				TEST 2				SUBJECT	TEST 1				TEST 2			
	TUBE 1		TUBE 2		TUBE 1		TUBE 2			TUBE 1		TUBE 2		TUBE 1		TUBE 2	
	MIN.	SEC.	MIN.	SEC.	MIN.	SEC.	MIN.	SEC.		MIN.	SEC.	MIN.	SEC.	MIN.	SEC.	MIN.	SEC.
1	2	15	2	15	2	35	2	20	26	3	00	3	00	3	15	3	45
2	2	30	2	30	2	30	2	45	27	4	15	4	15	4	30	4	15
3	3	30	3	25	3	15	3	30	28	5	00	4	30	5	00	4	45
4	3	45	4	00	4	00	3	50	29	4	15	4	30	4	30	4	15
5	4	00	4	30	4	15	4	45	30	4	00	4	10	4	00	4	15
6	4	30	4	15	4	15	4	15	31	3	30	3	45	4	00	3	30
7	4	00	4	20	4	30	4	30	32	4	00	4	00	4	15	4	30
8	4	25	4	15	4	30	4	30	33	5	00	5	15	5	15	5	00
9	3	20	3	30	3	30	3	15	34	5	30	5	30	5	30	5	30
10	3	45	4	00	4	00	4	10	35	5	00	4	45	4	45	4	30
11	4	00	4	15	4	10	3	50	36	4	30	4	45	4	15	4	30
12	3	30	3	30	3	30	3	30	37	5	30	5	15	5	15	5	15
13	4	00	4	20	4	30	4	30	38	3	30	3	15	3	15	3	00
14	3	20	3	00	3	10	3	10	39	2	45	2	30	2	50	2	40
15	5	00	5	00	4	45	4	45	40	2	15	2	30	2	00	2	15
16	4	10	4	00	4	15	4	20	41	4	00	4	10	3	45	4	00
17	3	00	3	10	3	20	3	00	42	3	40	3	15	3	45	3	40
18	4	10	4	00	4	00	3	50	43	4	10	4	30	4	30	4	15
19	4	30	4	30	4	30	4	15	44	4	00	4	00	4	20	4	40
20	3	50	4	00	4	00	3	45	45	3	45	4	00	4	00	4	30
21	3	30	3	45	3	15	3	45	46	4	10	4	05	3	45	3	30
22	4	10	4	30	4	30	4	30	47	2	45	3	10	2	40	3	00
23	4	00	3	45	3	45	3	45	48	4	10	3	50	3	45	4	10
24	2	25	2	45	2	45	2	45	49	3	20	3	30	3	00	3	20
25	3	40	3	50	3	20	3	20	50	3	45	3	50	3	45	3	30

*Each test was made in duplicate.

been chosen because it approximates room temperature and can therefore be readily maintained.

RESULTS

In Table I are given the results of tests performed on fifty normal individuals. Each test was done in duplicate and was immediately repeated. Note that with one exception (Subject 44) the greatest variation in the coagulation time of consecutive samples from any one individual was thirty seconds or less. The shortest coagulation time in this series was two minutes, fifteen seconds, and the longest five minutes, thirty seconds at 25° C. Because of the reproducibility of the method, it is felt that these variations represent true differences in the coagulation time of these normal people. Both the median and the average of the coagulation time in this series were close to four minutes. In Table II are compared the results obtained by this method with the standard Lee-White venepuncture procedure. The Lee-White method records the time necessary for relatively complete clotting to occur, whereas this method, because of the delicacy of the end point, indicates the time necessary for the initiation of coagulation. This undoubtedly explains the difference in normal coagulation times between the Lee-White and this method. In our experience the prolonged coagulation times observed after the administration of heparin also may be readily obtained by this method.

TABLE II. COMPARISON OF THE CAPILLARY TUBE METHOD WITH THE LEE-WHITE PROCEDURE*

SUBJECT	CAPILLARY TUBE						LEE-WHITE		
	TUBE 1		TUBE 2		AVERAGE		TUBE 1	TUBE 2	TUBE 3
	MIN.	SEC.	MIN.	SEC.	MIN.	SEC.	MIN.	MIN.	MIN.
1	4	10	4	45	4	28	8	11	13
2	4	10	4	25	4	17	9	10	12
3	2	45	3	00	2	52	10	12	13
4	4	10	3	45	4	27	10	11	12
5	3	20	3	30	3	25	10	12	13
6	3	45	3	50	3	48	10	11	12
7	4	10	4	00	4	05	12	12	13
8	3	30	3	40	3	35	11	12	13
9	4	05	4	20	4	12	10	12	13
10	3	00	3	20	3	10	9	11	12

*Tubes 1 and 2 in the Lee-White column included only for completeness, column 3 being the coagulation time as indicated by that method. As noted in the text, this capillary tube method indicates the initiation of coagulation, whereas the Lee-White method indicates relatively complete coagulation.

SUMMARY

A simple method of determining the coagulation time using capillary blood is presented; normal values with this method ranged from two minutes, fifteen seconds to five minutes, thirty seconds at 25° C. with a variation in duplicate tests of thirty seconds or less. Both the median and the average coagulation time in this series were very close to four minutes.

REFERENCES

1. de Takats, Geza: Heparin Tolerance, A Test of the Clotting Mechanism, Surg., Gynec. & Obst. **77**: 31, 1943.
2. Loewe, L., Rosenblatt, P., Greene, H. J., and Russel, M.: Combined Penicillin and Heparin Therapy of Subacute Bacterial Endocarditis, J. A. M. A. **124**: 144, 1944.
3. Kolmer, J. A.: Clinical Diagnosis by Laboratory Examinations, New York, 1943, D. Appleton-Century Co., Inc., p. 38.
4. Todd, J. C., and Sanford, A. H.: Clinical Diagnosis by Laboratory Methods, ed. 10, Philadelphia, 1944, W. B. Saunders Co., p. 197.
5. Lee, R. I., and White, P. D.: A Clinical Study of the Coagulation Time of Blood, Am. J. M. Sc. **145**: 495, 1913.

A SIMPLE PROCEDURE FOR DETERMINING THE APPROXIMATE CONCENTRATION OF PEPSIN IN GASTRIC CONTENTS

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NEW YORK, N. Y.

THE method to be described is a simplification of the procedure described by Barowsky, Tauber, and Kleiner.[1] The same basic principle is utilized, namely, the milk-clotting action of gastric fluid at pH 5.0. There is no rennin in human gastric juice; at any rate, not in that of the adult.[2] Therefore, a measure of the milk-clotting power is a measure of peptic activity.

The difference between the procedure mentioned and the present one is that in the former the greatest dilution of gastric juice which produces a clot is determined, while in this modification the length of time required for a single tube to clot is ascertained. The other, and more accurate procedure, requires the preparation of ten or more dilutions. The objection to this is the time consumed in preparing these dilutions, particularly if great accuracy is not required. Only one dilution and one portion of buffered milk are used in the simplified method. The length of time required for clotting in this single test varies inversely with the amount of pepsin present (Table I).

TABLE I. INTERPRETATION OF RESULTS

CLOTTING TIME (MIN.)	TENTATIVE COMPARISON WITH NORMAL	APPROXIMATE NUMBER OF UNITS/C.C. GASTRIC JUICE	SUGGESTED METHOD OF REPORTING
Less than 3	Very high	Over 2,500	++++
3 to 4	Moderately high	2,001-2,500	+++
4 to 10	Usual normal	1,001-2,000	++
10 to 15	Low	501-1,000	+
Over 15	None to trace	0-500	0 or trace

Solutions Required.—

A. Buffer. Dissolve 42 Gm. of NaOH in about 500 c.c. distilled water; add 115 c.c. 80 per cent acetic acid; dilute to 1,000 c.c. This keeps well.

B. Fresh cow's milk.

Equal volumes of A and B are mixed well; 10 c.c. are needed for each test.

Procedure.—

1. Centrifuge or filter gastric contents, if necessary.
2. Test reaction with Congo red paper.
3. (a) If acid, dilute 1 c.c. to 50 c.c. with water. Mix well.
   (b) If not acid, dilute a small quantity accurately with an equal volume of 0.1 N HCl; mix well and then dilute 1 c.c. to 25 c.c. with water. Mix again.
4. Place a test tube containing 10 c.c. of buffered milk in water at 20° C.

From the Department of Physiology and Biochemistry, New York Medical College, Flower and Fifth Avenue Hospitals.

Demonstrated at the Graduate Fortnight, New York Academy of Medicine, Oct. 11-22, 1943.

Received for publication, May 11, 1945.

5. Add 1 c.c. of the diluted gastric fluid to the test tube of milk, mix quickly, replace in the water bath, and note the time.

6. Note the time when the milk clots (i.e., either thickens on tilting the tube or small white particles separate out).

REFERENCES

1. Barowsky, H., Tauber, H., and Kleiner, I. S.: An Office Procedure for the Rapid Estimation of Pepsin and Its Clinical Significance, Am. J. Digest. Dis. & Nutrition **4**: 229, 1937.
2. Dotti, L. B., and Kleiner, I. S.: The Absence of Rennin From Adult Human Gastric Juice, Am. J. Physiol. **138**: 557, 1943.

BOOK REVIEWS

Essentials of Allergy. By *Leo H. Criep*, M.D., Assistant Professor of Medicine and Lecturer in Immunology, School of Medicine, University of Pittsburgh; Diplomate in Internal Medicine (1937); Fellow of the American Academy of Allergy; Chairman Department of Medicine, Montefiore Hospital and Senior Staff in Medicine, Presbyterian and Passavant Hospitals, Pittsburgh; Consultant in Allergy to the Medical Service of the United States Veterans Administration. J. B. Lippincott Company, Philadelphia. Price $5.00. Cloth with 381 pages and 42 illustrations.

Contents: 1. Hypersensitiveness. 2. Anaphylaxis. 3. Allergy. 4. Diagnosis of allergy. 5. Treatment of allergy. 6. Pollen allergy (Hay fever). 7. Bronchial asthma. 8. Nasal allergy. 9. Skin allergy (Allergic dermatoses). 10. Serum allergy. 11. Drug allergy. 12. Bacterial allergy. 13. Fungus allergy. 14. Physical allergy. 15. Miscellaneous allergy. 16. Allergy in children. 17. Diagnostic skin tests.

The author has succeeded in condensing into a small volume a great deal of the present-day knowledge of clinical allergy. The book contains much useful information for undergraduate medical students and practicing physicians, for whom it is primarily intended. The information is, in general, authoritative, based upon modern clinical standards in a rapidly developing field. Numerous case reports are presented. The subject matter is concise and presented without controversial discussion. The thoughtful, analytical reader, however, will find several minor inaccuracies, together with several misleading and confusing statements. The following are called to attention:

1. On the inside of the front cover anaphylaxis is defined as "a state of hypersensitiveness produced experimentally in animals by injecting them for the second time with the same protein." (On page 2 we find that by "animals" is meant only "a lower animal"). In reality the "state of hypersensitiveness" is frequently, and indeed usually, produced by the *first* injection. It is the reaction or shock which is produced by the *second* (or subsequent) injection.

2. The author has followed the custom, which is prevalent in certain quarters, of applying the term "anaphylaxis" to the hypersensitiveness produced in guinea pigs, rabbits, dogs, etc., by injections of certain foreign substances (e.g., sera). But when these same substances are injected into another species of animal, namely, man, the resulting hypersensitiveness is called "allergy." This, the reviewer believes, is an artificial and misleading separation of analogous (if not identical) phenomena. The issue is further confused by the classification of "serum allergy" as item IIB under "acquired allergy" as distinct from item A-anaphylaxis, yet on page 260 we find that in ordinary serum sickness "precipitins last for several days to a few weeks . . ." and "anaphylactic antibodies usually accompany them."

3. The terms "acquired" and "familial" are used in a confusing manner. There may be, and in fact there is, a familial tendency to acquire certain allergies. But the allergy is, in fact, acquired at some time during the life of the individual—usually after birth, occasionally during intrauterine life. No allergy has been proved to be other than "acquired."

4. On page 15, paragraph 2, there appears the following statement: "There is no evidence to indicate that it is possible to sensitize human beings artificially regardless of whether they are normal or atopic." This sentence is incorrect as stated and is, in fact, contradicted in the following paragraph in which it is admitted that injections of horse serum may result in skin sensitivity and even in the development of atopic reagins.

5. On page 84 may be found recommendations for strengths of extracts used for intradermal testing, including fish and eggs in "1-10 dilution." The reviewer considers this strength decidedly unsafe (unless preceded by scratch tests).

6. In the discussion of "atopic or familial (allergic) dermatitis" beginning on page 226, the statement is made that the allergen is brought to the skin by the hematogenous route and is usually a food although it may be an inhalant. The shock tissues are said to

be the endothelial cells lining the skin capillaries, and the diagnostic test is given as the scratch or intradermal test. While this is probably the prevailing view among allergists, there are some dissenting voices, including that of Dr. R. L. Cooke, who wrote the foreword to the book. These dissenting voices will probably increase greatly in number with the passage of time. These dissenters have reasons for believing that, in this disease, the hematogenous route is not important in the transportation of allergen to the skin, that the allergens are not often foods or inhalants, and that scratch and intradermal tests are not often of diagnostic value. The author, however, probably does well to avoid such controversial issues in a book of this nature.

F. A. S.

Clinical Roentgenology of the Digestive Tract. By *Maurice Feldman*, M.D., Assistant Professor of Gastroenterology, University of Maryland; Assistant in Gastroenterology, Mercy Hospital; Consulting Roentgenologist, Sinai Hospital; Consulting Roentgenologist, Sinai Hospital. Second edition. Williams & Wilkins Company, Baltimore, Md., 1945. Price $7.00. Cloth with 769 pages.

Dr. Feldman's book will be a very useful reference for students and practitioners. The clinical factors of etiology, incidence of disease, age distributions, symptoms, and pathology are adequately stated for their use in roentgenological interpretation. The descriptions of the roentgenological pictures are concise and full and give differential details. The discussions given of many rare diseases and conditions of the gastrointestinal tract with careful statement of their rarity should be especially valuable to students and casual users of the x-ray in diagnosis. For experienced and specialized gastrointestinal roentgenologists the book gives an informative orientation to the study of the tract and its appendages. Only in reference to the illustrations may they find more than personal differences. More illustrations would be helpful to the less experienced and to beginners in the specialty. The bibliography is not extensive but well selected from world sources and adequately pertinent to the nonreview presentation. The author has very well achieved his object in presenting a general guide to the roentgenological diagnosis of the digestive tract and in indicating its importance and value. The book will be valuable to all who may be interested, either casually or especially, in gastroenterology and should find a place in their library.

Studies of Burns and Scalds (Reports of the Burns Unit, Royal Infirmary, Glasgow, 1942-1943). Medical Research Council, Privy Council, Special Report Series No. 249. Price, 4s. Od. net. Hard paper cover with 209 pages.

In this excellent clinical report 127 cases are summarized, thirty in considerable detail. Observations include many chemical studies as well as a variety of clinical data. The data recorded will certainly prove of great interest to those who are studying burns clinically, and many of the observations will be a stimulus to those interested in a few of the many unsolved problems. The local treatment is simple and consists of gentle cleansing with a detergent, followed by the application of a dressing over a sulfonamide cream. Systemic therapy consists of plasma and serum transfusions in large amounts.

An interesting and important experiment was carried out in four patients in whom a green dye was injected intravenously to determine the extent of the increased capillary permeability. It was demonstrated in at least one patient that the center and therefore the more severely burned area remained unstained by the dye. This obviously indicated a type of burn which had destroyed the circulation through the skin and was therefore associated with less extravasation. This observation perhaps is not sufficiently emphasized. It has an important bearing on the general assumption that the amount of fluid lost in a burn and therefore the amount required to correct hemoconcentration varies roughly with the extent of the burn. It seems clear that this would apply only if the entire burned area were associated with extravasation. This would be more likely when the thermal injury did not coagulate the skin, for example, in scalds. Flame burns, on the other hand, might destroy the circulation through the injured area and thus limit the degree of exudation. Such differences may explain some of the discrepancies between the degree of hemoconcentration and the extent of the burn which the authors noted. These considerations emphasize the need for describing the type of burn present in each case. The title of the monograph suggests that the cases would be so classified, but this promise is not fulfilled. It might have been profitable to correlate the degree of hemoconcentration with the kind of thermal injury (that is, scald or burn) as well as with its extent. A very instructive series of photographs shows the rapidity

with which edema of the face subsides spontaneously after a superficial burn. Twenty-four hours after the injury the patient's facial edema, which nine hours before prevented the eyes from opening, had almost completely subsided.

Intravenous injections were carried out rather extensively, the fluid being largely serum or plasma. In some cases concentrated serum was used. The amounts injected were large and by present standards might be called excessive. The indications for the use of intravenous fluids were at first based on the development of hemoconcentration or other evidence of need. Soon this plan was changed to one in which plasma or serum was given early and more or less as a routine in order to *prevent* hemoconcentration. This latter plan was said to be justified by the results, which, however, on analysis seemed equivocal. Indeed, the authors admit that at least one patient had an excessive amount of intravenous fluid. For example, one 6-year-old child with a burn involving but 10 per cent of the body was given intravenously 1,900 c.c. (50 c.c./kg.) of plasma during six hours.

Anemia was observed only in the more serious cases. Red blood cell levels about 50 per cent of normal were reached in practically all of the patients with the most extensive burns on the tenth to the fourteenth day. In an attempt to explain this anemia, studies were made of the fragility of the red cells. Significant alterations were observed in the response of the red cells in burn patients as compared with the fragility of the red cells in normal subjects subjected to the same test. Moreover, many patients showed gross hemolysis in their circulating blood and a few contained hemoglobin in the urine. All patients showing hemolysis in the plasma in large amounts were very extensively and severely burned and all except one died within twelve hours of injury.

The notable contribution of the Glasgow School is the demonstration that infection can be greatly reduced if not almost eliminated by meticulous attention to the fundamental principles of aseptic surgery. Although the authors believe that their use of local sulfonamide therapy was partly responsible, the evidence indicates that the outstanding reason for their successful control of infection was a consistent drive in the handling of the cases against contamination and cross infection. This required painstaking attention to detail, careful masking of the patient and personnel, complete sterilization of everything coming into contact with the burn, and even systematic elimination of air-borne infection from dust in the room, on the floor, and by movement of personnel. It was quite evident from their experience that such detailed though simple measures definitely resulted in a tremendous decrease in the incidence of infection. This experience emphasizes the fundamental fact already demonstrated during World War II, that chemotherapy will not of itself prevent or eliminate infection and that in the case of most surgical wounds, fundamental principles of surgical technique demand precedence and emphasis. Chemotherapy can be effective only as an adjunct or adjuvant, not as a substitute for these principles.

Introduction to Animal Biology. By *John B. Parker*, Ph.D., Professor Emeritus of Biology, Catholic University of America, Washington, D. C.; and *John L. Clarke*, Ph.D., Assistant Professor of Biology, Retired, Catholic University of America, Washington, D. C. Second Edition. The C. V. Mosby Company, St. Louis, 1945. Price $3.75. Cloth with 532 pages.